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

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Targeting stromal interactions in the pro-metastatic tumor microenvironment

Endoglin & TGF-β as (un)usual suspects

Madelon Paauwe

Targeting stromal interactions in the pro-metastatic tumor microenvironment – *Endoglin & TGF-β as (un)usual suspects*
Madelon Paauwe

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Targeting stromal interactions in the pro-metastatic tumor microenvironment

Endoglin & TGF-β as (un)usual suspects

PROEFSCHRIFT

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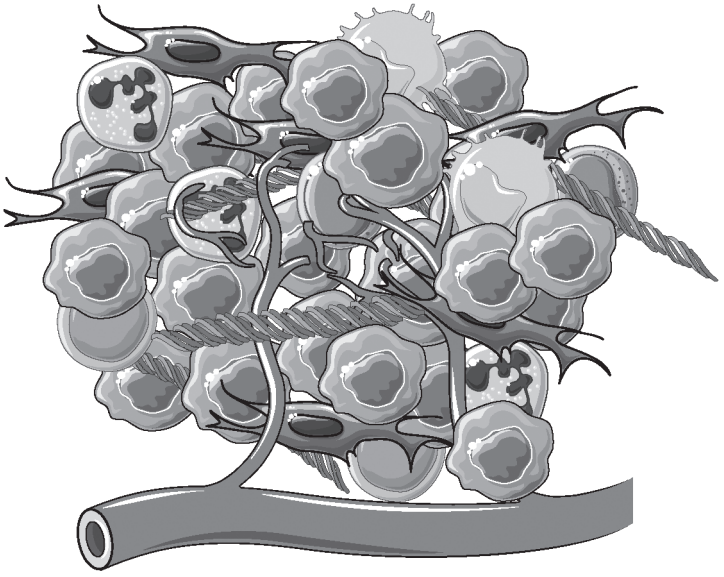
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Een wetenschapper in een laboratorium is niet enkel een technicus:
hij is ook een kind dat door natuurverschijnselen geboeid wordt als
door een sprookje
- *Marie Curie*

Madame Curie: A Biography (1937)

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Chapter 1

General introduction

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1. Cancer

Despite continuous efforts in the past decades to unravel the molecular mechanisms underlying cancer initiation and progression and to translate findings into preventive and therapeutic strategies in order to reduce cancer mortality and morbidity rates, overall cancer mortality remains 8.2 million per year worldwide (*1*). This number accounts for almost 15% of worldwide deaths (*1, 2*), occupying second place in the top ten of leading causes of death (*3*). Cancer mortality is mainly caused by metastatic disease, rather than the primary tumor. Although mortality rates are decreasing, cancer incidence is rising (*4*) and is expected to keep rising in the next two decades (*5*). This increase is partly due to improved diagnostics and population screening efforts, but nevertheless reflects increased cancer burden and underlines the need for more effective anti-cancer strategies.

1.1. Breast cancer

As the most commonly diagnosed cancer in women, breast cancer accounts for 6.4% of total cancer deaths worldwide (*1*). Risk factors for breast cancer development include hereditary mutations, age, obesity, smoking and hormonal changes (e.g. pregnancy or menopause). Breast tumors are staged from 0 to 4, depending on tumor size, invasiveness, lymph node involvement and metastatic spread (*6*). Based on molecular characteristics, three main subtypes of breast cancer have been established (*7*). First, the luminal tumors are the most common breast cancer subtype and are characterized by high expression of estrogen receptor (ER) and progesterone receptor (PR) and generally have a good prognosis (*8-10*). Second, basal-like tumors are generally negative for ER, PR and human epidermal growth factor receptor 2 (HER2), therefore often referred to as triple negative (*8*). This subtype is particularly aggressive and has a poor prognosis (*11*). The third subtype consists of HER2 overexpressing tumors, which are generally negative for both ER and PR (*12*). Although these tumors usually respond well to treatment, relapse rates are high resulting in a poor prognosis (*9, 10, 13*). Primary treatment for all subtypes of breast cancer is surgical removal of the primary tumor, complemented with adjuvant chemo-, radiation- or hormonal therapies (*14*).

1.2. Colorectal cancer

Representing 8.5% of all cancer deaths, colorectal cancer (CRC) represents a significant proportion of cancer-related deaths. This third most common type of cancer is slightly more common in men than in women and appears mostly in developed countries (*4*). CRC risk factors include age, obesity and smoking, but also Western-type diets have been shown to increase CRC risk. Most colorectal cancers arise from premalignant polyps (adenomas) by acquiring additional genetic mutations in the epithelial cells. Subsequent mutations result in growth of non-invasive carcinoma *in situ,* which eventually progress to invasive tumors, which then progress through different stages (Fig. 1). Colorectal tumors are staged at diagnosis using the TNM classification (*15*), which is shown in Table 1. As for

breast cancer, the primary treatment for CRC is surgical removal of the tumor. Dependent on tumor staging, this therapy is complemented with pre- or post-surgical radiation or chemotherapy. Stage III and IV CRC patients receive adjuvant chemotherapy or radiation, whereas for stage I and II CRC patients, adjuvant treatment is not typical (*16*).

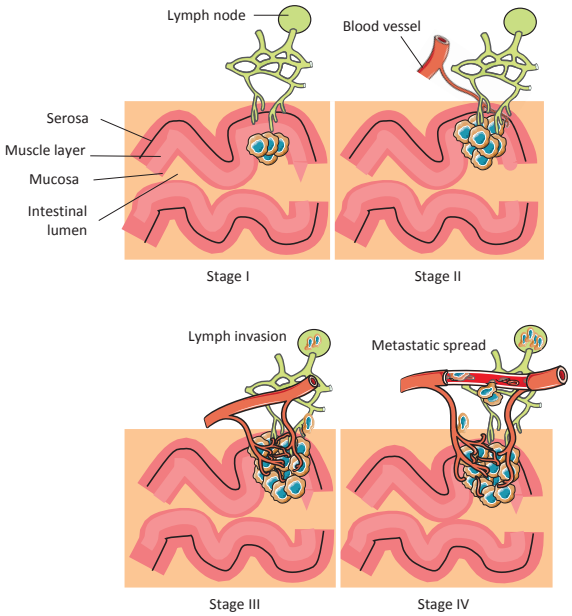


Figure 1. Schematic representation of the different stages of CRC development. Stage-I is characterized by the formation of a small tumor mass which invades through the intestinal mucosa into the muscle layer. During progression to stage-II CRC, angiogenesis is induced and tumor cells start to invade the serosa. Stage-III is marked by lymph node involvement, whereas metastatic spread to distant organs is one of the characteristics of stage-IV CRC.

Table 1. Colorectal cancer staging

Stage	Characteristics
0	Carcinoma *in situ*
I	Tumor invades colonic mucosa and muscular layer, no lymph nodes involved
II	Tumor invades through intestinal wall, no lymph nodes involved
III	Tumor invades through intestinal wall, one or multiple lymph nodes involved
IV	Tumor has spread to distant one or more distant sites of the body

2. Tumor microenvironment

Cancerous growth starts from genetic mutations in epithelial cells, which allow unlimited proliferation and activation of growth stimulatory pathways (*17*). Therefore, initial cancer therapies were focused on targeting the mutated epithelial cells. This approach resulted in therapies which showed initial response, but because of the high mutation rate in epithelial cells, tumors rapidly became resistant to therapy. One molecular pathway in which the genes encoding signaling transducers are frequently mutated in epithelial tumor cells is the transforming growth factor (TGF)-β signaling pathway.
In recent years, the tissue surrounding the tumor, also called the tumor microenvironment (TME) or stroma, has been recognized to play an important role in tumor progression and metastasis (*18*). The TME consists of endothelial cells, establishing the tumors' vascular system, immune cells, cancer-associated fibroblasts (CAFs) and extracellular matrix (*19*) (Fig. 2). The total stromal compartment has been shown to be an independent factor predicting patient survival, at least in CRC and certain breast cancer subtypes (*20-22*). Since the realization that the TME plays a pivotal role in cancer progression, a number of drugs have been developed targeting specific components of the TME. This thesis focuses on the role of specific TME cellular subsets in tumor progression and metastasis and their potential for therapeutic targeting. One of the pathways affecting almost all components of the TME is the TGF-β signaling pathway.

TGF-β signaling plays a dual role in cancer due to its cancer stage-dependent effects on, amongst others, epithelial tumor cells. The TGF-β family consists of more than 30 structurally and functionally related family members and is extensively involved in almost all processes in the human body (*23*). Members include the TGF-βs, bone morphogenetic proteins (BMPs) and activins. The role of TGF-β signaling in embryonic development and tissue homeostasis has been extensively researched and discussed in detail (*24, 25*). Different TGF-β family members bind to distinct receptors, but canonical signaling is generally induced by similar mechanisms. Signaling is initiated by binding of the ligand to its type II receptor, which subsequently recruits and transphosphorylates the type-I receptor. As a result, downstream receptor-regulated Smads are activated by phosphorylation. Upon complex formation with the co-Smad, i.e. Smad4, these dimeric or trimeric complexes can translocate to the nucleus and regulate gene transcriptional responses (*26*). Which genes are induced or repressed is highly dependent on cellular context, for example on which receptors or downstream Smads become activated and which Smad interacting transcription factors are present (*27, 28*). Although TGF-β was first recognized as a growth factor, stimulating fibroblast proliferation (*29*), it has become clear that in normal tissues, TGF-β mainly functions as an anti-proliferative factor. In cancer, TGF-β displays a dual effect. In early stages of cancer, TGF-β inhibits tumor cell proliferation, whereas in more advanced tumors TGF-β functions as a tumor promoting cytokine (*30*), both via effects on the epithelial tumor cells as well as on other components of the TME. Although tissue specific

inactivation of TGF-β signaling components often does not result in spontaneous tumor formation (*31-33*), its important tumor suppressive role becomes apparent upon tissue damage or oncogenic stimuli, as shown in models for CRC (*32, 34*) and breast cancer (*35*). Several components of the TGF-β signaling pathway are mutated and inactivated in cancer, being one mechanism by which TGF-β loses its inhibitory effect on cell proliferation. One of the tumor promoting actions of TGF-β is the induction of epithelial to mesenchymal transition (EMT) (*36, 37*), which aids in tumor cell invasiveness and therefore increases metastatic capacity (*38-40*). Moreover, TGF-β can promote tumor cell proliferation by inducing the production of mitogenic factors by the tumor cells themselves (*41*). The role of TGF-β in metastasis has been intensely studied and it appears that its role in facilitating metastasis is highly tumor- and context-dependent. For example, TGF-β affects the ability of breast cancer cells to metastasize to the lung or bone, although through two different mechanisms (*42-44*). A role for TGF-β in preparing and maintaining the metastatic niche was reported, for example in bone metastasizing breast cancer (*45*). Also, the ability of TGF-β to induce factors stimulating angiogenesis aids in outgrowth of distant metastases (*46*). Finally, other TME components are also affected by TGF-β, for example resulting in increased production and modulation of extracellular matrix by CAFs or suppression of the immune system.

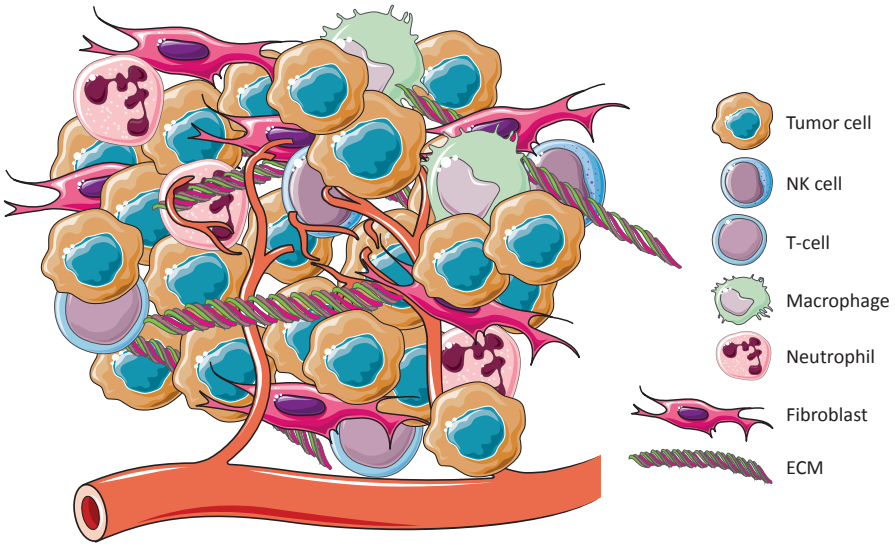


Figure 2. The tumor microenvironment. Epithelial tumor cells are surrounded by the tumor microenvironment which exists of endothelial cells, fibroblasts, extracellular matrix and a variety of infiltrated immune cells.

Although TGF-β signaling is involved in almost all physiological processes, many efforts have been made to target this pathway in cancer. Clinical and preclinical studies have investigated the effects of interrupting TGF-β/TGF-β receptor interaction by antibodies and ligand traps,

inhibiting TGF-β at gene expression level and by blocking TGF-β receptor activity using kinase inhibitors (*47*). Preclinical data showed that, although TGF-β targeting had a limited effect on epithelial cell proliferation (*48-50*) tumor inhibiting effects were accomplished by affecting the TME, like fibroblasts (*51, 52*), endothelial cells (*53-56*) and immune cells (*57-67*). Additionally, simultaneous targeting of TGF-β receptors I and II resulted in decreased metastatic spread of CRC cells in a mouse model for CRC metastasis (*68*). Multiple phase I and some phase II clinical studies have been performed, mainly showing increased immune response upon targeting TGF-β signaling (*69-71*) and some cases of disease stabilization (*69, 70, 72*) and improved survival were observed (NCT01246986). In addition, clinical trials targeting TGF-β specifically in tumor vessel formation are ongoing, based on the crucial role of TGF-β in this process.

2.1. Endothelial cells

Endothelial cells form all vascular structures in the body and play an important role in tumor progression by regulating blood and nutrient supply, removal of waste products, secretion of cytokines and attracting immune cells (*73, 74*). Solid tumors require the formation of their own vascular network (angiogenesis) to provide nutrient and oxygen supply in order to sustain tumor growth (*73*). Additionally, the newly formed vessels provide a route of dissemination to other organs, rendering angiogenesis indispensable for metastatic spread (*75*). Based on these characteristics, anti-angiogenic therapies have been widely used in treatment of solid tumors, including breast- and colorectal cancer. In the early 1970's Judah Folkman already discovered that when tumors grow beyond a few mm^3, the tumor core becomes hypoxic. As a response, epithelial tumor cells start producing pro-angiogenic factors, which lead to proliferation and differentiation of endothelial cells, ultimately resulting in increased tumor vessel density (*75*) (Fig. 3). Pro-angiogenic factors secreted by tumor cells include vascular endothelial growth factor (VEGF) (*75*), TGF-β family members TGF-β and bone morphogenic protein (BMP)-9 (*76, 77*), and IL-8 (*78-80*).

2.1.1. VEGF

VEGF was recognized as a major pro-angiogenic factor, being indispensable for both developmental and tumor angiogenesis and was therefore the first factor that was therapeutically targeted in cancer therapy (*75, 81, 82*). Although results from preclinical studies using the first anti-VEGF antibody bevacizumab (Avastin®) looked very promising, efficacy in the clinic as a monotherapy proved to be limited. This lead to withdrawal of Food and Drug Administration approval for bevacizumab as adjuvant treatment in metastatic HER2-negative breast cancer (*83*). In CRC, bevacizumab remains approved for treatment of metastatic disease in combination with various regimens of chemotherapy, including 5FU-based therapy (*84*). Additional to antibodies targeting pro-angiogenic factors or their receptors, kinase inhibitors that target receptor activity have been developed. For example, SU5416, which is a VEGF receptor kinase inhibitor, was tested in clinical trials as an adjuvant treatment in CRC. However, the phase III clinical trial was stopped due to a lack

of clinical benefit (NCT000212810). One of the main reasons for limited clinical effects is the development of therapy resistance, characterized by initial therapeutic response (e.g. tumor shrinkage), followed by disease progression (*85*). Therapy resistance can occur by upregulation of alternative pro-angiogenic pathways (*86*), like the TGF-β signaling route.

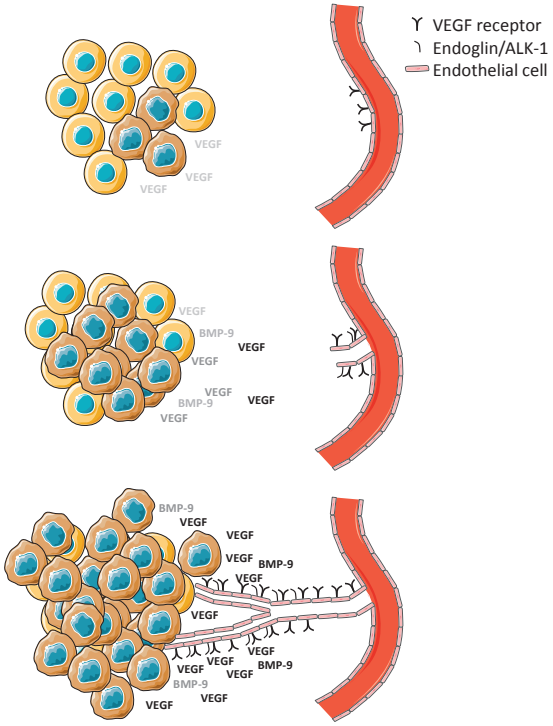


Figure 3. The process of tumor angiogenesis. In response to hypoxia, due to tumor growth, tumor cells start to secrete pro-angiogenic factors like VEGF and BMP-9. Endothelial cells sense increased concentrations of VEGF or BMP-9 with the VEGF receptor II or endoglin/ALK1, respectively. As a result, endothelial cells proliferate and migrate towards the increased concentration and establish novel tumor vasculature.

2.1.2. Endoglin & ALK-1

Angiogenic processes are also stimulated by TGF-β signaling, contributing to tumor growth and tumor cell dissemination (*87*). In endothelial cells, TGF-β signaling can occur through heteromeric complex formation of the TGF-β receptor II (TGFβRII) with TGF-β type I receptors, activin receptor-like kinase (ALK)-5 or ALK-1 (*88*). Recruitment of ALK-5 receptor inhibits angiogenesis, while ALK-1 stimulates an angiogenic response (*89*). Although TGF-β ligands can signal directly through TGFβRII and ALK-1, expression of the TGF-β co-receptor endoglin potentiates this signaling pathway (*90, 91*) and enhances the angiogenic response by increasing endothelial cell proliferation and migration (*92*). Moreover, the presence of endoglin also inhibits the anti-angiogenic effects of TGF-β (*90, 93*). BMP-9 can bind directly to both ALK-1 and endoglin, without the recruitment of additional BMP receptors (*94, 95*). The importance of ALK-1 and endoglin in angiogenesis is underlined by

embryonic lethality upon knock out (*96-99*) and the formation of vascular malformations in endoglin heterozygote mice and humans (Reviewed in (*100*) and discussed in chapter 3). Additionally, in cancer an increased number of vessels expressing endoglin correlates with poor patient survival in various solid tumors (*101*). Also, ALK-1 expression has been shown to correlate to metastatic disease in breast cancer patients (*102*). Based on their importance in angiogenesis, both ALK-1 and endoglin are considered to be promising targets in anti-angiogenic therapy and are therefore developed clinically. In order to prevent therapy resistance, simultaneous targeting of multiple pro-angiogenic pathways could pose an effective method and is therefore currently being explored in clinical trials (e.g. NCT01648348, NCT01727089, NCT01975519, NCT01306058, NCT01806064).

One strategy to target ALK-1-mediated signaling is the use of ALK-1-Fc. This fusion protein consists of the extracellular domain of ALK-1 fused to the Fc-part of an antibody and functions as a ligand trap, thereby preventing BMP-9 binding to ALK-1 and inhibits angiogenesis (*103*). Pre-clinical studies have shown that this strategy decreased tumor angiogenesis in a model for pancreatic cancer and inhibited metastatic spread in a mouse breast cancer model (*102, 103*). Another approach, which is under clinical development, is the use of anti-ALK-1 antibodies (*104*). These antibodies prevent ligand-induced receptor complex formation between endoglin and ALK-1 (*105*), and thereby inhibit angiogenic signaling.

In addition to ALK-1, endoglin targeting is being explored as a target for anti-angiogenic therapy. Endoglin targeting can be accomplished by using an endoglin-Fc fusion protein, sequestering endoglin ligands. Furthermore, an endoglin neutralizing antibody, TRC105, is being developed by Tracon Pharmaceuticals. This antibody specifically inhibits binding of the endoglin ligand BMP-9 (*106*). Additionally, TRC105 induces apoptosis of targeted cells through antibody-dependent cytotoxicity. This antibody is currently under development in phase II clinical studies as anti-angiogenic therapy in metastatic disease for various tumor types (NCT01375569, NCT01090765, NCT01328574, NCT01381861, NCT01778530). Underlying the importance of endoglin expression, Anderberg et al. showed that endoglin heterozygosity disrupted vascular integrity, thereby enhancing the formation of breast cancer metastases *in vivo (107)*. Therefore, the occurrence of metastatic spread should be closely monitored during therapeutic endoglin targeting.

2.2. Cancer-associated fibroblasts

Next to endothelial cells, cancer-associated fibroblasts (CAFs) comprise a major part of the TME and have been shown to interact with malignant cells and other stromal cells (*108, 109*). Due to the lack of one specific marker to identify CAFs, the generally accepted marker is α-Smooth Muscle Actin (αSMA) expression (*110*), although αSMA-negative CAFs also exist (*111*). In both breast cancer and CRC, the percentage of αSMA-positive tumor stroma was shown to be an independent factor in predicting prognosis (*112, 113*). However, it was recognized in the past decade that different subsets of CAFs exist, which cannot be distinguished solely based on αSMA expression (*114, 115*). The difference in CAF subsets

can partly be ascribed to different cells of origin of CAFs and by the differential expression of cell surface markers, which affect CAF function. As can be expected, there are tumor-promoting and -restraining effects of CAFs, partly explained by the existence of different subsets and environmental context (*116*).

2.2.1. Origin of CAFs

As mentioned above, several hypotheses exist on the cell type of origin of CAFs. The most widely accepted hypothesis describes the differentiation of local fibroblasts and tissue resident fibroblast precursors into CAFs (*117*). Tumor cells produce a wide variety of cytokines, of which TGF-β is highly involved in the differentiation of local fibroblasts into CAFs (*118, 119*). However, these cytokines also have long-ranging effects that play a role in attracting bone marrow-derived cells (BMCs) which have the ability to differentiate into CAFs (*120-124*). Additionally, BMCs have the ability to attract local fibroblasts which, once in the tumor, adopt a CAF phenotype (*125*). Mesenchymal stem cells (MSCs) have been suggested as an important BMC in this process, based on their ability to differentiate into various stromal cell types (*126, 127*). Next to recruitment of cells, proliferation of myofibroblasts already present in the tissue can be a source for CAFs (*128*). Another hypothesis is based on the process of epithelial-to-mesenchymal transition in which tumor cells differentiate from epithelial cells into cells with mesenchymal characteristics, showing overlap with CAF-like properties (*129*). This same process has also been described for endothelial cells (EndMT), resulting in stromal cells expressing both αSMA and the endothelial marker CD31 (*130*). Finally, smooth muscle cells covering blood vessels (pericytes) have also been suggested as a cell of origin for CAFs (*131*). Based on the wide variety of CAF sources, this partly explains the heterogeneity in CAF subsets.

2.2.2. Tumor promoting effects

CAFs play an important role in tumor initiation, progression and metastasis by a variety of mechanisms (Fig. 4). The role of CAFs in tumor initiation has been demonstrated by introducing genetic changes specifically in fibroblasts, which resulted in tumor formation. For example, Bhowmick et al. knocked out TGFβRII specifically in fibroblasts and found spontaneous tumor formation in mouse prostate and stomach (*132*). Additionally, fibroblast-specific deletion of the BMP receptor II was reported to induce epithelial hyperplasia in the mouse colon (*133*). This does not only underline the role of fibroblasts in tissue homeostasis and tumor initiation, but also implies an important role for TGF-β/BMP signaling in tumor formation. More than two decades ago, the importance of CAFs in tumor progression was recognized when tumor cells derived from fibroblast-rich tumors proved to be more invasive in diverse *in vitro* assays than the parental cells (*134*). The mechanism underlying pro-tumorigenic effects of CAFs was further investigated and paracrine signaling between tumor cells and fibroblasts proved to be a major factor. As an example, CAF-secreted proteases can enhance tumor invasion (*135*). Moreover, *in vitro* it has been shown that fibroblast-mediated invasion of CRC cells is dependent on TGF-β (*136*). These

results were confirmed *in vivo* by Calon et al., who showed that TGF-β production by CRC cells induced a pro-metastatic gene profile in CAFs and resulted in increased metastatic spread. Additionally, inhibition of TGF-β signaling diminished the number of metastases in various mouse models for CRC (*137*).
CAFs secrete a wide variety of cytokines and chemokines that highly affect both the tumor cells and the tumor stroma. When CRC cells, *in vitro* or *in vivo,* were stimulated with CAF conditioned medium, migration and invasion of tumor cells increased (*138, 139*). Moreover, CAF-derived extracellular matrices were shown to stimulate breast cancer cell spread *in vitro*, suggesting a role in metastasis (*140*). Additional to direct interactions with tumor epithelial cells, CAFs secrete large amounts of proteases and extracellular matrix. As a consequence, matrix-bound VEGF is released and activated, leading to increased tumor angiogenesis and ultimately contributing to tumor progression and metastasis (*141*). Moreover, remodeling of the extracellular matrix leads to decreased matrix – tumor cell interaction and a physically more invasive matrix, thereby increasing tumor metastasis (*142*). Additional to matrix remodeling, it has been observed in squamous cell carcinoma that CAFs not only create a path through the ECM, but also physically lead cancer cell invasion (*142*).

Besides (in)direct effects on epithelial tumor cells, CAFs also play an important role in regulating the immune status of solid tumors (*143, 144*). For example, Kraman et al. showed that upon depletion of a CAF subset expressing fibroblast activation protein (FAP), established lung cancers showed rapid immune-dependent tumor reduction (*145*). Mechanistic studies have been performed to identify specific factors and signaling pathways in CAF – immune cell interaction. This research showed that CAFs express a broad variety of pro-inflammatory chemokines and cytokines involved in recruitment of various immune cells (reviewed in (*146*)). Although CAFs secrete pro-inflammatory chemokines, attracting immune cells, they drive the differentiation of these cells to an anti-inflammatory state once infiltrated in the tumor (*147*). This immunosuppressive differentiation is induced by CAF excreted factors (*148*). Moreover, CAFs physically surround tumor cells with extracellular matrix, thereby protecting them from immunogenic recognition (*149*).
Based on these tumor promoting properties, CAFs pose a promising target in treating cancer. Different clinical studies have already assessed the possibility of targeting CAFs by inhibiting FAP activity in various solid tumors (*150, 151*). Currently, more phase I clinical trials are ongoing in patients with metastatic CRC (NCT00004042) and other advanced solid tumors (NCT02558140, NCT02627274).

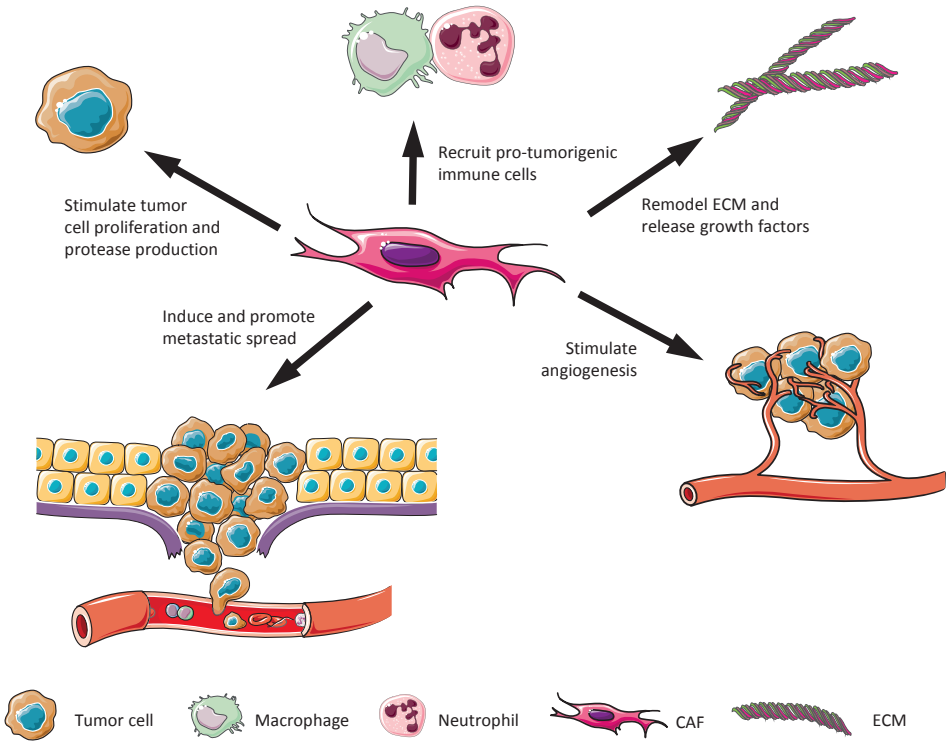


Figure 4. Pro-tumorigenic interactions of CAFs. CAFs stimulate tumor progression and metastasis by interacting with all cell types in the TME and by remodeling the extracellular matrix (ECM).

2.3. Immune cells

The final component of the TME to be discussed are immune cells. Initially, immune infiltration in a tumor was viewed as an attempt of the body to attack and destroy the tumor (*152*). This hypothesis was strengthened by the observation of increased tumor burden in immunodeficient mice, when compared with immunocompetent animals (*153, 154*). Additionally, high infiltration of cytotoxic T-cells (CTL) and natural killer (NK) was correlated to improved survival in CRC (*155*).

However, more recent research has shown that immune infiltrate can also enhance tumorigenesis and progression (*147, 156-158*). Tumors have been described as "wounds that do not heal" (*159*), due to their resemblance to chronically inflamed wounds, which are characterized by persistent presence of inflammatory cells, in particular macrophages and neutrophils, that are essential in wound healing (*160*). In a tumor though, this persistently inflamed state allows the tumor to grow and progress by the stimulatory factors secreted by these immune cells (*161*). Additional to subverting the inflammation process, tumor cells also secrete factors which suppress the function of anti-tumor CTLs and NK cells (*162, 163*). Also recruitment of immunosuppressive cells, like regulatory T-cells, aid in creating

a tumor promoting microenvironment (*164*). In this process, TGF-β functions as a potent immunosuppressor by inhibiting pro-inflammatory and stimulating immunosuppressive cellular subsets (Reviewed in (*165*)).

Although B- and T-cells play important roles in tumor progression (*166, 167*) and their targeting is currently clinically applied, these cells will not be discussed in detail in this thesis. Macrophages and neutrophils in particular have been shown to play important roles in creating a tumor-promoting environment and will therefore be discussed in more detail below and illustrated in figure 5.

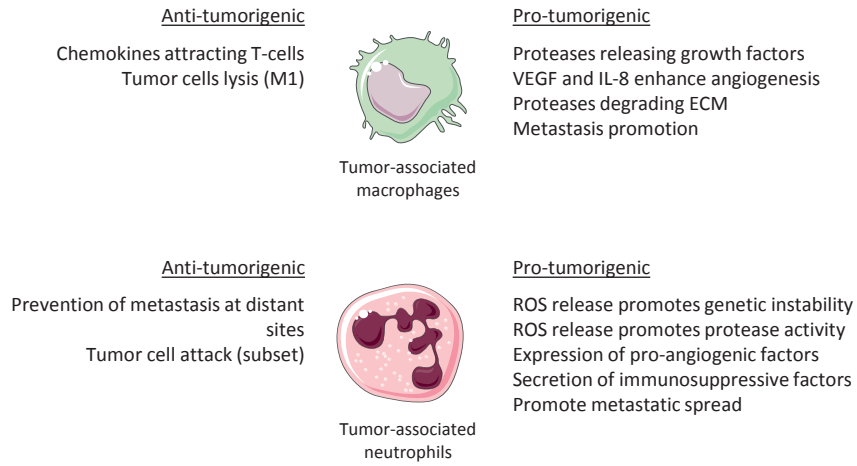


Figure 5. Pro- and anti-tumorigenic properties of macrophages and neutrophils. Macrophages and neutrophils are often observed in solid tumors and were reported to have both pro- and anti-tumor effects.

2.3.1. Macrophages

Tumors secrete various chemokines in order to recruit monocytes from the circulation and to initiate macrophage differentiation (*168-172*). Expression of specific chemokines and cytokines promotes monocyte differentiation into tumor promoting M2 macrophages, rather than the anti-tumor M1 macrophage subset. M2 macrophages produce chemokines involved in regulatory T-cell recruitment and promote wound healing, angiogenesis and tissue remodeling (*173*). Tumor-associated macrophages (TAMs), which resemble the M2 subtype, are known to express several proteases, which play an important role in tumor progression by releasing growth factors from the extracellular matrix (*174*). Additionally, macrophages secrete the pro-angiogenic cytokines VEGF and interleukine-8 (IL-8) (*175, 176*). TAM-secreted proteases not only enhance angiogenesis, but by degrading the extracellular matrix, also play an important role in tumor progression through the release of growth factors (*177, 178*). Finally, the presence of macrophages has been shown to correlate with the metastatic potential of various tumors, including breast cancers (*179-181*). These observations were further strengthened by a study in which lung metastasis in a mouse breast cancer model was reduced upon macrophage depletion (*182*). Based on their

tumor promoting characteristics, therapeutic targeting of macrophage subpopulations is being clinically explored in diverse solid tumors, including CRC and breast cancer (e.g. NCT02448810, NCT00262808 and NCT00257322).

2.3.2. Neutrophils

The role of neutrophils in cancer growth and progression is less well characterized, but in recent years this area of research has evolved. The tumor promoting effects of neutrophils are mediated by the release of reactive oxygen species (ROS) which enhance the genetic instability of cancer cells (*183, 184*), and enhances the activity of the protease matrix metalloproteinase (MMP)-9, promoting tumor cell invasion (*185*). Also, secretion of a wide variety of pro-angiogenic factors contributes to the tumor progressive effect of neutrophils (*186, 187*). A role for neutrophils in metastasis has also been shown in multiple publications. For example, paracrine interactions between breast cancer cells and neutrophils resulted in increased VEGF production by tumor cells, contributing to tumor cell invasiveness *in vitro (188)*. Moreover, in a mouse model for melanoma recruited neutrophils have been shown to enhance metastatic spread to the lungs (*189*). However, studies reporting opposite findings suggest anti-metastatic effects of neutrophils (*190*) and show a role for TGF-β in differentiation of neutrophils into pro- or anti-tumorigenic subsets (*191*). The important role of neutrophils in colorectal cancer was highlighted by depletion studies in a chemically-induced mouse model for CRC in which neutrophil depletion resulted in decreased tumor number and size (*192, 193*). Therapeutic targeting of neutrophils and neutrophil function is currently being explored in pre-clinical and clinical research, for both breast- and colorectal cancer.

3. Endoglin on non-endothelial cells

As described above endoglin is highly expressed on activated endothelial cells the TME. Additionally, limited studies have described endoglin expression on other cell types, where it influences the invasive or migratory capacity of these cells.

Endoglin has been reported to be expressed by some epithelial (tumor) cells. For example, keratinocytes have been shown to express endoglin under both physiologic and pathologic conditions (*194*). In an animal model for skin cancer, endoglin heterozygosity lead to reduced growth of skin lesions, however the progression to malignant carcinomas was increased (*194*). Reduced endoglin expression decreased proliferation, but increased EMT in keratinocytes (*195*), suggesting that endoglin has a dual role in cancer, as was reported for TGF-β. Endoglin expression has also been observed in various human breast cell lines. One of these being the non-tumorigenic MCF10, which showed increased *in vitro* invasion upon oncogene activation, when endoglin was knocked down (*196*). Moreover, expression of endoglin in endogenously endoglin-negative breast cancer cells, reduced invasion and metastasis *in vivo*. Finally, in clinical samples, endoglin expression on breast cancer cells

was found to correlate to improved patient survival (*196*). Another study showed that endoglin expression in esophageal squamous carcinoma cells is decreased by epigenetic silencing. Overexpression of endoglin in cell lines led to less invasion and decreased tumor growth when engrafted *in vivo (197)*. Endoglin is also expressed by Ewing sarcoma cells and in Ewing sarcoma patients, expression of endoglin is associated with poor patient survival (*198*). Finally, one study showed that endoglin is expressed in uterine leiomyosarcomas, where it correlates with poor patient survival, increased migration and invasion. Based on the limited number of studies on endoglin on epithelial cells, it appears that its role is both tumor type- and context-dependent.

An important role for endoglin was reported in the recruitment of tumor promoting CAFs in prostate cancer models. Endoglin is highly expressed on these CAFs and crucial for their survival (*199*). Endoglin heterozygosity in this model decreased metastatic potential, while it increased primary tumor growth (*199*). Interestingly, tumors in endoglin heterozygous mice comprised significantly less CAFs than their wild type controls. *In vitro*, conditioned medium from endoglin heterozygous fibroblasts reduced cancer cell migration, when compared with controls (*199*).

Although it appears that endoglin expression on CAFs might play a role in tumor metastasis, additional research has to be performed.

4. *In vitro* models for cell-cell interactions

The pro-tumorigenic interaction between tumor cells and the different cell types in the TME is of high importance for tumor growth and metastasis. Therefore, therapies targeting these interactions are (pre-)clinically explored. In order to evaluate these interactions *in vitro*, a broad range of co-culture models have been established. One experimental possibility is to use conditioned medium from one cell type to stimulate the second cell type and thereby mimic paracrine interactions. To study the effect of direct cell-cell contact culturing of both cell types of interest in mixed co-cultures is an often used method (*200*). However, this model is not suitable to distinguish effects of these interactions in the individual cell types (*201*).

Effects of paracrine signaling or inhibition of signaling pathway components, can be assessed using different experimental set-ups, dependent on the cell type of interest.

4.1. *In vitro* angiogenesis

The angiogenic properties of endothelial cells can be analyzed *in vitro* by 2- and 3-dimensional assays assessing tube formation or endothelial sprouting. Importantly, the extracellular matrix which forms the basement membrane on which endothelial cells grow *in vivo* is highly involved in both *in vitro* assays (*202, 203*).

In the 2-dimensional cord formation assay, endothelial cells are seeded onto a matrix-coated surface and tube formation is rapidly induced in the presence of angiogenic growth

factors (*204*). Angiogenic capacity can be quantified by different methods, including counting the number of branches per branch point or the quantification of the number of closed polygons (*205*). This model takes most steps of angiogenesis into account, however, the 3-dimensional structure in which angiogenesis occurs *in vivo* is not adequately modeled by this system. Therefore, the use of 3-dimensional endothelial spheroids is often used as a more complex model for angiogenesis (*203*). Endothelial spheroids are formed on methocell-coated surfaces which results in formation of multicellular aggregates (*206*). Spheroids are subsequently embedded in a collagen-I matrix, which inhibits endothelial cell proliferation, and endothelial tube formation is induced by angiogenic factors, like VEGF (*207, 208*). This model more closely resembles the angiogenic process *in vivo* than the tube formation assay. Both *in vitro* models proved to be a useful tool in screening of novel anti-angiogenic drugs and pose a good intermediate between monolayer cell culture and *in vivo* experiments.

4.2. CAF invasion *in vitro*

As for the process of angiogenesis, CAF invasion can be studied *in vitro* using different models, differing in level of complexity. To study chemotaxis-mediated invasion, the Boyden chamber is often used (*209*). In this assay, cells are seeded on top of a matrix-coated membrane and left to migrate towards a gradient of chemoattractant or conditioned medium for a fixed duration. At the end of the experiment, invaded cells are quantified and stimulating or inhibiting effects on invasive behavior can be assessed. A more complex invasion assay, taking the extracellular matrix and 3-dimensional conformation into account, is the spheroid invasion model. Closely resembling the endothelial sprouting assay on a technical level, this model determines fibroblast invasion into a collagen-I matrix. The use of stimulating ligands and inhibitory antibodies can be tested in this model, and even conditioned medium can be used to assess the effects on fibroblast invasion. The use of co-cultures of fibroblast with epithelial cancer cells has been reported to study the pro-invasive role of fibroblasts in cancer (*210, 211*). Co-culture spheroids can be used to study tumor biology and to closely investigate targeting of cell-cell interactions in an *in vitro* system that resembles *in vivo* situations, thereby posing a valuable tool in cancer research (*212*).

5. Zebrafish models in cancer

As a model system between complex *in vitro* experiments and mouse studies, zebrafish models for cancer have emerged in the past two decades (*213*). Zebrafish show high homology with humans on both the genetic and physiologic levels (*214-219*) and approximately 70% of all genes involved in human disease have homologs in zebrafish (*220*). Additionally, the rapid development of the zebrafish and the relatively inexpensive housing and food render zebrafish a suitable model for large scale drug screening and modeling of

human disease development, including cancer. One frequently studied process of cancer development in zebrafish is tumor angiogenesis. Anti-angiogenic drug delivery can be achieved through direct injection into the zebrafish embryos or by supplementing the water of the fish. The translucency of zebrafish embryos and the availability of a transgenic zebrafish line with green fluorescent protein (GFP) expressing vasculature allow easy monitoring of anti-angiogenic effects using confocal microscopy (*221-223*). Additionally, these properties make it relatively easy to follow the spread of fluorescently labeled tumor cells and zebrafish are therefore often used as a model for metastasis (*221, 224*). However, there are some drawbacks of the use of zebrafish in cancer research, which include the presence of multiple copies of a gene in zebrafish, complicating complete deletion. Also the lower temperature of fish maintenance (28°C) may impact on human cells and the administration of water-insoluble drugs might hamper drug delivery (*213, 225*). Finally, zebrafish embryos lack a functional immune system, further complicating accurate analysis of therapeutic efficacy (*213*).

6. Mouse models

To study the complicated communication patterns involving direct cell-cell contact and paracrine signaling without excluding any involved cell type, matrix component or cellular interaction, *in vivo* cancer models are used (*226*). Different mouse strains exist, all with their own characteristics, e.g. allowing for different tumor induction methods or the use of human cells in mice. Although the information obtained from animal studies is very valuable, a one-on-one translation to clinical effects is hardly ever feasible and should always be considered when implementing pre-clinical data in clinical studies. For the studies in this thesis, several different mouse models were used of which two will be discussed in more detail.

6.1 Orthotopic breast cancer model

Breast cancer researchers use different methods to implant tumors, of which subcutaneous injection is a widely used model. However, the use of orthotopic breast cancer models, in which tumor cells are transplanted to the organ of origin, take the original TME into account and have been shown to more efficiently promote tumor progression and metastasis than subcutaneous models for breast cancer (Reviewed in (*227*)). In our experiments, invasive lobular breast cancer was studied in an orthotopic transplantation model, using mouse breast cancer cells. The mouse breast cancer cell line KEP1-11 (*228*) was used to circumvent interspecies complications and to assure interaction between tumor cells and the TME. Balb/c nude mice, which lack a thymus and are therefore immunodeficient, showed most efficient tumor take and were therefore used in our studies. Therefore, any effects of the adaptive immune system cannot be assessed using this mouse strain. However, the innate immune system is present and shows normal activity.

In our experiments breast tumors were induced by exposure of the fourth mammary fat pad by a small incision in the skin (Fig. 6A) and injection of luciferase-expressing KEP1-11 cells. Tumor growth was assessed by bioluminescent imaging (Fig. 6B). To closely mimic clinical situations, we used a breast cancer resection model in which primary tumors are surgically removed (Fig. 6C). As in the clinic, in this model treatment starts after tumor resection and metastatic spread is followed over time using bioluminescence (Fig. 6D). Using this model, we have assessed tumor angiogenesis and metastatic spread of breast tumors and delivery of chemotherapeutic agents.

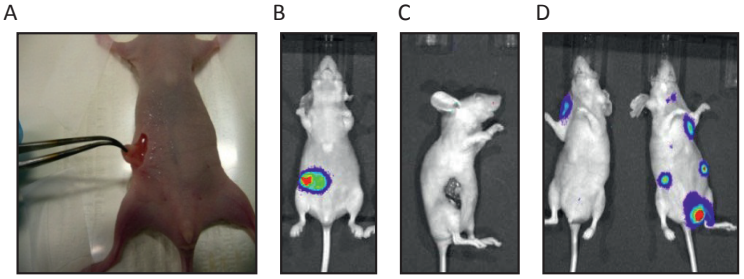


Figure 6. Orthotopic breast cancer model. Mouse breast cancer cells are transplanted to the mammary fat pad (A), and tumor growth is monitored by bioluminescence (B). For the resection model, the primary tumor was resected after four weeks (C) and metastatic spread was followed over time (D).

6.2 Colorectal cancer model

Different *in vivo* models for CRC cancer have been developed, each with their own advantages and limitations. Four main models can be distinguished (*229*); 1. Genetic models in which germ line mutations result in spontaneous tumor formation. 2. Xenotransplant models in which human CRC tissue is used in immunodeficient mice. 3. Syngeneic transplantation of mouse tumor tissue to immunocompetent mice. 4. Chemically induced models in which carcinogens are used to induce tumor growth. The advantages of chemically induced models include relatively short tumor induction period and the resemblance to some characteristics of human CRC (*229*).

The carcinogen azoxymethane (AOM) has been described to induce colorectal tumors, which share histopathological characteristics with human CRC (*230, 231*). In combination with dextran sodium sulphate (DSS), AOM-induced tumor formation was shown to be highly accelerated (*231*). The protocol of our model included one intraperitoneal injection with AOM, followed by three 21-day cycles of DSS. During the experiment, mice develop colitis, resulting in weight loss, rectal blood loss and occasionally rectal prolapse (Fig. 7A). Mild colitis-induced symptoms disappeared during the "off" period of the cycles. Tumor initiation and growth in this model can be assessed using mouse endoscopy as shown in figure 7B, allowing for monitoring of tumor growth in time. Both gross morphology and histochemical analysis at the end of our experiments readily showed tumor formation in

the colorectum (Fig. 7C). This chemically induced model allows for efficient *in vivo* studies of CRC, taking human tumor characteristics into account.

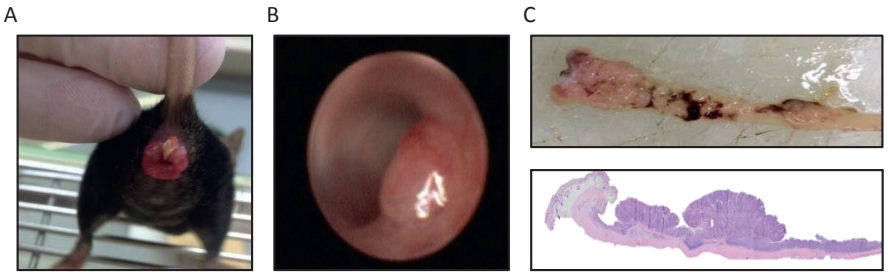


Figure 7. Chemically-induced mouse model for CRC. Colorectal tumor growth is induced by AOM/DSS treatment. During DSS cycles mice suffer from colitis, which, in combination with tumor growth, results in rectal blood loss and occasionally rectal prolapse (A). Tumor growth in this model can be monitored using mouse endoscopy (B, T indicates tumor). Upon termination of the experiment, tumor formation was clearly visible on both gross morphology and histochemical analysis.

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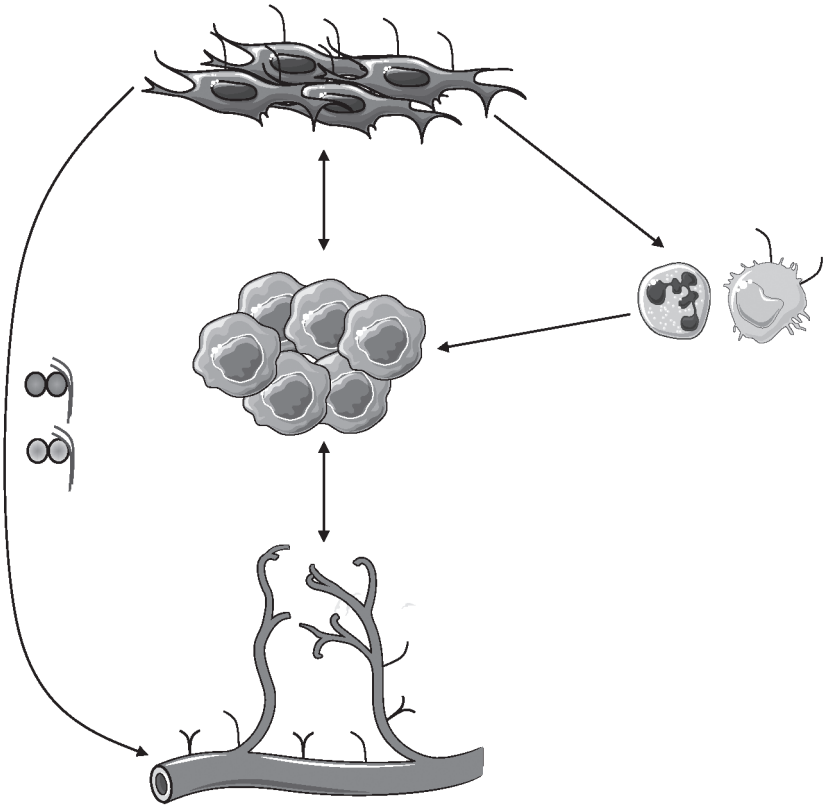
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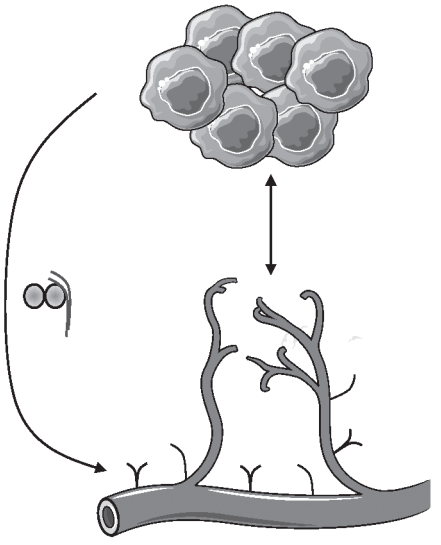
Chapter 2

Thesis aim and outline

The interaction between epithelial tumor cells and their microenvironment is of crucial importance in cancer progression. Both direct cell-cell contact and paracrine signaling, as well as more long distant effects of cytokines and chemokines are involved in tumor progression and metastatic spread. Therefore, the tumor microenvironment (TME) has been recognized an important player in cancer progression and its potential as a therapeutic target is extensively explored. This thesis describes studies on the transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP)-mediated interactions between tumor cells and specific TME components and evaluates their potential as a therapeutic target in either breast- or colorectal cancer (CRC). In patients, we have observed changed TGF-β/BMP signaling in both tumor cells and the TME in various solid tumors. To study TGF-β/BMP-mediated interactions between tumor cells and stromal cells, we have developed *in vitro* and *in vivo* models systems. The aim of this thesis is to unravel the pro-tumorigenic role of TGF-β/BMP-mediated interactions in the TME, with a specific interest in the co-receptor endoglin, and assess their potential as a therapeutic target.

In **chapter 3**, the potential of using endoglin as a target for tumor imaging and anti-angiogenic therapies is extensively discussed. Endoglin expression on endothelial cells will be the main focus of this review, although other cell types are also discussed. **Chapter 4** decribes studies on the anti-angiogenic properties of ALK1-Fc, an ALK1 ligand trap. The results show, improved tumor vessel quality and therefore better delivery of chemotherapeutic agents in several different solid tumors. Combined targeting of the pro-angiogenic vascular endothelial growth factor (VEGF) and TGF-β/endoglin signaling pathways in breast cancer is discussed in **chapter 5**. This study illustrated, using both *in vitro* and *in vivo* models, that dual targeting of pro-angiogenic pathways decreased angiogenesis to a higher extent than targeting both pathways separately. More importantly targeting of endoglin, using either the neutralizing antibody TRC105 or and endoglin ligand trap, decreased metastatic spread in a mouse model for breast cancer. TGF-β-mediated interactions between cancer-associated fibroblasts (CAFs) and epithelial tumor cells will be described in **chapter 6**. This study showed a cancer-promoting feedback loop which is the result of hyperactivated TGF-β signaling in CAFs, leading to increased TGF-β production and protease secretion, which ultimately leads to increased invasive behavior.

The role of endoglin on CAFs is more extensively researched in CRC, described in **chapter 7**. Endoglin expression on CAFs at the invasive border of colorectal tumors proved to be a predictive factor for metastatic disease in early stage CRC. *In vitro* and *in vivo* studies described in this chapter show a role for endoglin in CAF invasion and endoglin appears to be involved in CRC metastasis. Finally, **chapter 8** reports on the fibroblast-specific role of endoglin in CRC which was assessed in a mouse model for chemically-induced CRC. Fibroblast-specific knock out of endoglin resulted in enhanced tumorigenesis in this model, accompanied by expansion of the stromal tumor component and increased infiltration of specific immune cells subsets. In **chapter 9**, the data of the different studies are summarized and discussed.



Chapter 3

Endoglin for tumor imaging and targeted cancer therapy

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Abstract

Introduction: Although cancer treatment has evolved substantially in the past decades, cancer-related mortality rates are still increasing. Therapies targeting tumor-angiogenesis, crucial for the growth of solid tumors, mainly target vascular endothelial growth factor (VEGF) and have been clinically applied during the last decade. However, these therapies have not met high expectations, which were based on therapeutic efficacy in animal models. This can partly be explained by the upregulation of alternative angiogenic pathways. Therefore, additional therapies targeting other pro-angiogenic pathways are needed.

Areas covered: The transforming growth factor (TGF)-β signaling pathway plays an important role in (tumor-) angiogenesis. Therefore, components of this pathway are interesting candidates for anti-angiogenic therapy. Endoglin, a co-receptor for various TGF-β family members, is specifically overexpressed in tumor vessels and endoglin expression is associated with metastasis and patient survival. Therefore, endoglin might be a good candidate for anti-angiogenic therapy. In this review we discuss the potential of using endoglin to target the tumor vasculature for imaging and therapeutic purposes.

Expert opinion: Considering the promising results from various *in vitro* studies, *in vivo* animal models and the first clinical trial targeting endoglin, we are convinced that endoglin is a valuable tool for the diagnosis, visualization and ultimately treatment of solid cancers.

1. Introduction

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths in 2008 and although multiple new (targeted) therapies have been approved, cancer mortality is estimated to continue to rise to 13.1 million deaths in 2030 [1]. Therefore, novel therapeutic strategies are needed, accompanied by advanced imaging techniques to visualize tumors at an early stage.

Tumor growth beyond a diameter of 3-4 mm is critically dependent on angiogenesis to deliver oxygen and nutrients to, and remove CO_2 and waste products from tumor cells [2]. Besides delivery of nutrients and oxygen, a vascular network provides a route for disseminating tumor cells [3]. Tumor angiogenesis seems to be an attractive target for both imaging and therapeutic strategies. In 1971, vascular endothelial growth factor (VEGF) was identified as a potent factor in inducing tumor angiogenesis [4] and was explored as the first target for anti-angiogenic therapy. Antibody-based anti-VEGF therapies were developed, which showed strong anti-tumor efficacy in various *in vivo* mouse models for cancer [5]. However, the effectiveness of anti-VEGF therapy in the clinic proved to be strongly tumor type dependent and effects were mostly transient, due to resistance. Therefore, these therapies did not meet the high expectations of increasing patient survival in many tumor types and even led to the withdrawal of FDA approval for anti-VEGF therapy in breast cancer [6].

One of the major mechanisms of resistance to anti-VEGF therapy is the upregulation of alternative pro-angiogenic pathways [7], opening the possibility to explore these pathways to serve as novel anti-angiogenesis targets. Among several others, the transforming growth factor-β (TGF-β) pathway plays a prominent role in tumor-angiogenesis. TGF-β is the prototype of a large family of structurally and functionally related cytokines, which include the TGF-βs, bone morphogenetic proteins (BMPs) and activins. The role of the multifunctional cytokine TGF-β seems to be dual and dependent on the cancer stage [8]. TGF-β suppresses tumor growth during the premalignant and early malignant stages, whereas at later stages this inhibitory function is lost. Moreover, in late stages TGF-β acts as a tumor promoter by stimulating cancer cell invasion, immune suppression and inducing angiogenesis [9]. TGF-β induces tumor angiogenesis by affecting both endothelial cells (ECs) and perivascular cells [10, 11]. Therefore, TGF-β seems to be an attractive target for cancer therapy and clinical trials targeting TGF-β pathway ligands or receptors are ongoing [12]. The direct pro-angiogenic effects of TGF-β on ECs are mediated via the TGF-β co-receptor endoglin. Endoglin is highly expressed on proliferating ECs [13-15]. Furthermore, high microvessel density, determined by endoglin expression, has been correlated with poor prognosis in many tumor types [16-18]. Therefore, endoglin appears to be an attractive target for anti-angiogenic therapy in cancer patients. Additionally, the specific expression of endoglin in angiogenic vessels [11, 19, 20] could also render it a suitable candidate for tumor imaging. In this review we focus on the prognostic value of endoglin in cancer, the potential role of targeting endoglin for imaging the tumor vasculature and targeting endoglin for cancer therapy.

2. Endoglin structure and function

2.1 Endoglin structure

The endoglin gene is located on the long arm of human chromosome 9 [21] and is composed of exons 1 to 8, 9A and 9B and 11 to 14 [22-24]. The promoter showed strong and selective activity in ECs, when compared to other cell types [25-28]. Human endoglin is a 658 amino acid long protein containing a large extracellular, a single transmembrane and a short cytoplasmic domain [29] (Fig.1). High sequence homology is found in the cytoplasmic and transmembrane regions of human, porcine and murine endoglin proteins. The extracellular domains are mostly divergent, but do contain highly conserved regions [30, 31]. Endoglin is expressed on the cell surface as a disulfide-linked 180 kDa dimer [29, 32-34] and functions as a co-receptor for TGF-β1 and -β3 [35]. Endoglin is also able to interact with BMP-2, -7 and -9 [36, 37].

Betaglycan, the other TGF-β co-receptor, is structurally related to endoglin, mainly in the transmembrane and intracellular domains [38, 39], while the extracellular domain demonstrates less than 30% homology [38]. Endoglin can bind BMP-9 in the absence of the type-II TGF-β receptor (TβRII) [40], but requires the oligomerization with TβRII to bind TGF-β1 and –β3. Betaglycan, on the other hand, is able to bind all isoforms of TGF-β independent of TβRII [41, 42]. In general, endoglin and betaglycan form homodimers, but betaglycan/endoglin heterodimers have also been reported, although their function remains unclear [43].

The extracellular domain of endoglin contains an orphan and zona pellucida (ZP) domain [29, 44, 45] (Fig.1). The latter domain comprises an arginine-glycine-aspartic acid (RGD) motif [29, 44, 45], which may function as an interaction site for integrins and was recently suggested to be crucial for leukocyte adhesion and transmigration [46]. The RGD motif is present in human but not in mouse endoglin [29]. The ZP domain is involved in endoglin oligomerization and the interaction with TβRII and the type-I TGF-β receptor (TβRI) ALK-5 [47]. The endoglin cytoplasmic domain contains many serine and threonine residues, of which specific residues are phosphorylated by TβRII, ALK-5 and ALK-1 [48-50]. Endoglin expression is upregulated by both TGF-β and hypoxia [51]. A hypoxia-responsive element (HRE) was identified downstream of the endoglin promoter, which can bind the hypoxia-inducible factor (HIF)-1α. Upon addition of TGF-β in hypoxic conditions, endoglin transcription increased markedly [51]. Endoglin expression on the cell surface can further be regulated by receptor shedding [52]. Membrane-type-1 matrix metalloprotease 1 (MT1-MMP or MMP14) cleaves endoglin from the membrane, releasing a soluble receptor, sEndoglin [53].

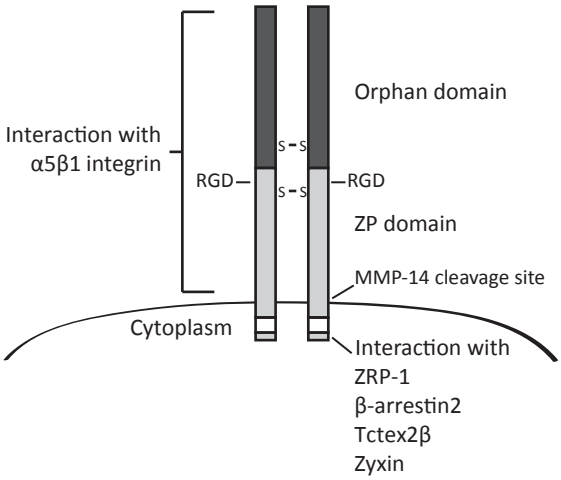


Figure 1 Schematic representation of endoglin structure. Endoglin is expressed at the cell surface as a disulfide-linked dimeric protein, consisting of a large extracellular, a single transmembrane and a short cytoplasmic domain. The extracellular region contains an orphan and a ZP domain, containing a RGD motif. ZP; zona pellucida, RGD; arginine-glycine-aspartic acid, MMP; matrix metalloproteinase.

2.2 Endoglin expression and function in endothelial cells and other cell types

High endoglin expression on vascular ECs has been reported at sites of active angiogenesis (e.g. during embryogenesis, in inflamed tissues and in solid tumors) [13-15]. In addition to ECs, endoglin is also expressed by other cell types. For example, endoglin is expressed by monocytes and its expression is further enhanced upon differentiation into macrophages [54]. During development, endoglin is required for myogenic differentiation [55], while during adulthood endoglin is expressed by hematopoietic stem cells [56], bone marrow stromal fibroblasts [20], melanocytes [57] and on the cell surface of syncytiotrophoblasts in term placenta [58]. Endoglin is also expressed by bone marrow-derived mesenchymal stem cells in which it plays a role in osteogenic differentiation [59]. Low endoglin expressing cells demonstrated increased osteogenic potential compared to high endoglin expressing cells [60]. Mancini and colleagues showed that in order for neural crest stem cells to undergo myogenic differentiation, endoglin is required [55]. Finally, during cardiac repair upon epithelial-to-mesenchymal transition of epicardium-derived stem cells endoglin expression is upregulated [61]. Endoglin is also expressed by a subpopulation of hematopoietic stem cells and can be used as a marker to define long-term repopulating hematopoietic stem cells [56, 62]. These studies indicate a potential role for endoglin in stem cell maintenance or differentiation of stem cells into different lineages. Further studies will be required to establish what the exact function of endoglin is during stem cell growth and differentiation. In pathological conditions, vascular smooth muscle cells inside atherosclerotic plaques were found to display high endoglin expression [63, 64]. After myocardial infarction, endoglin is highly expressed on mononuclear cells, where it has an indispensable role in leukocyte-mediated vascular

repair [65, 66]. In addition to cardiovascular diseases, sEndoglin levels are strongly increased during pre-eclampsia, a serious disease in pregnant women characterized by hypertension and proteinuria [63, 67]. Finally in cancer, besides very high expression on the vasculature, endoglin is also expressed by certain tumor cells including sarcoma and melanoma cells [68, 69]. Ovarian and prostate cancer cell lines were reported to express endoglin *in vitro*, but these results were not confirmed *in vivo* [70, 71]. In prostate cancer cells, endoglin seems to inhibit the migratory potential *in vitro* [72]. The differences in expression *in vitro* and *in vivo* could partly explain the contradictory findings which were reported on the endoglin in cancer.

2.3 Endoglin in developmental angiogenesis and hereditary hemorrhagic telangiectasia

The role of endoglin in developmental angiogenesis has been studied in genetically modified mice. Mice deficient in endoglin die around embryonic day E10.5 due to cardiovascular defects [73-75]. The phenotype observed in endoglin deficient embryos is comparable to that of knock-out animals for other components of the TGF-β signaling pathway, including TGF-β1, TβRII, ALK-1 and ALK-5 [76]. This indicates an indispensable role for the TGF-β signaling pathway in developmental angiogenesis. In humans, mutations in the endoglin gene have been described. It is generally accepted that endoglin haploinsufficiency results in the autosomal dominant syndrome hereditary hemorrhagic telangiectasia type 1 (HHT1) [22, 77-79]. HHT1 is characterized by telangiectasias, resulting primarily in epistaxis. Arterial venous malformations may also occur that may result in stroke, heart failure or fatal hemorrhage [77, 78]. In order to study HHT1 in an animal model, endoglin haploinsufficient ($Eng^{+/-}$) mice were generated, which are viable and manifest a phenotype similar to HHT1 patients, including vascular malformations and epistaxis [75]. Current treatment of HHT1 mainly focuses on reducing clinical symptoms. For example, Lebrin and coworkers showed that thalidomide treatment increases perivascular cell coverage in endoglin haploinsufficient mice. In HHT patients, thalidomide treatment reduced nose bleeds and nasal mucosal biopsies also suggested increased perivascular cell coverage [80], although fatigue and transient peripheral neuropathy were reported as side effects [81]. An alternative treatment to relieve HHT symptoms was evaluated by Albiñana and colleagues. This study showed that treatment of ECs with the estrogen receptor modulator raloxifene induced increases in both endoglin mRNA and protein accompanied by increased angiogenesis and EC migration. Following raloxifene treatment, HHT1 patients experienced less frequent epistaxis and did not experience serious side effects [82]. Currently, seven different clinical trials on HHT1 are ongoing. The majority of these trials focus on the use of the anti-VEGF antibody bevacizumab (National Clinical Trial number (NCT) NCT01397695, NCT01402531, NCT01314274, NCT01408030, NCT01507480) for inhibiting recurrent epistaxis. The other two trials, using thalidomide (NCT01485224) or sotradecol (NCT01408732), also aim to decrease the frequency and severity of epistaxis in HHT1 patients by increasing mural cell coverage.

2.4 TGF-β signaling and endoglin

TGF-β is synthesized as a latent precursor and can be activated via proteolytic cleavage or non-proteolytically through conformational changes [76]. Active TGF-β can bind to the TβRII, which recruits and transphosphorylates the TβRI, also called activin-receptor like kinase (ALK5, Fig.2). TβRI transduces the signal by phosphorylating the receptor-regulated Smads (R-Smads), which associate with the co-Smad (Smad-4 in mammals). This complex translocates to the nucleus, where it regulates transcriptional activity of target genes [83] (Fig. 2). Specific target genes are regulated, depending on which TβRI and intracellular Smads are involved. To date, 7 ALKs have been described, of which ALK-1 and ALK-5 are the type-I receptors expressed on ECs, where they play important roles during angiogenesis [84].

The role of TGF-β and ALK-1 and ALK-5 signaling in regulating EC function is complex. The apparent contradictory findings that have been reported for ALK-1 may be explained by the cellular context dependency of ALK-1 signaling, including the type of extracellular matrix surrounding cells [85-87]. In ECs, TGF-β can signal directly through TβRII and ALK-1 [88]. Expression of endoglin on ECs appears to be essential for efficient signaling [35] and induction of EC proliferation [88]. Furthermore, ALK-1 signaling stimulates angiogenesis, both directly and by inhibiting TGF-β/ALK-5 signaling, which is anti-angiogenic [87, 89, 90]. ALK-5 signaling was reported to be important for recruitment of perivascular cells and maturation of newly formed vessels [91]. Until recently, it was accepted that ALK-5 was indispensable for TGF-β/ALK-1 signaling [90], but Tian and colleagues showed that the presence of fibronectin can circumvent the necessity of ALK-5 in this signaling pathway [87].

In addition to being important in ECs, TGF-β signaling via the TβRII/ALK-1/endoglin pathway was also shown to be important in cardiac fibroblasts [92] and in tumor cells [83]. In addition to signaling via the canonical Smad pathway, endoglin is also able to induce downstream signaling in a Smad-independent manner. These pathways include MAP kinase signaling and the Rho-like GTPase and PI3K/AKT pathways [93]. The interaction with β-arrestin2 (see below) results in inhibition of MAPK signaling. In turn, this regulates vascular tone and permeability through interactions with eNOS [94].

Besides interactions with TGF-β ligands, endoglin also enables protein-protein interaction via its intracellular domain in a TGF-β-independent manner (Fig.1). For example, endoglin interacts with zyxin and ZRP-1, which are localized in focal adhesions and therefore could play a role in endoglin dependent cell migration [95, 96]. Additionally, interaction between β-arrestin2 and the intracellular domain of endoglin results in endosomal internalization of endoglin (Fig.1), decreased EC migration and inhibition of TGF-β-mediated ERK activation [97]. Moreover, the intracellular domain of endoglin interacts with Tctex2β, a protein involved in retrograde protein transport, thereby inhibiting transcriptional activity induced by TGF-β [90]. In ECs, endoglin has been shown to inhibit hypoxia mediated apoptosis. HIF-1α binds to the hypoxia responsive domain downstream of the endoglin promoter, resulting in upregulated endoglin expression [98]. Recently, the extracellular domain

of endoglin has also been shown to interact with α5- and β1- integrins, which induces endoglin/ALK-1 complex formation in the presence of fibronectin [87].

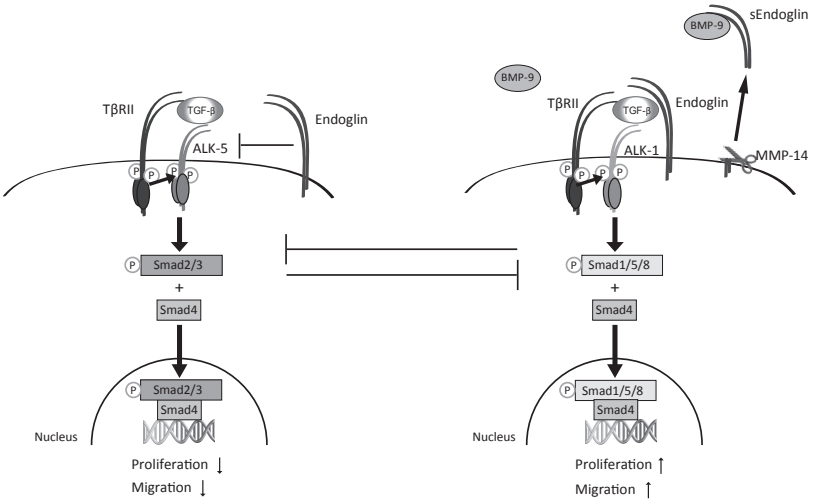


Figure 2 TGF-β signaling pathways in endothelial cells. In endothelial cells, TGF-β family members signal through different TβRII and TβRI combinations. TGF-β binds to TβRII and upon ALK-5 recruitment Smad-2/3 is phosphorylated. Activated Smad-2/3 proteins can form heteromeric complexes with Smad-4, followed by translocation and accumulation in the nucleus. When endoglin is present, TGF-β/ALK-5 signaling is inhibited and TGF-β signaling through ALK-1 is favored resulting in Smad-1/5/8 phosphorylation. Additionally, BMP-9 can bind to endoglin, also inducing Smad-1/5/8 phosphorylation. After complexation with Smad-4 these complexes accumulate in the nucleus, where pro-angiogenic transcriptional responses are initiated. Endoglin can be cleaved from the membrane causing membrane endoglin levels to decrease and soluble endoglin, which can function as a ligand trap, to increase.

3. The role of endoglin in disease

3.1 Fibrosis

TGF-β is known to play an important pro-fibrogenic role, for example, by increasing collagen deposition by fibroblasts in lung and liver fibrosis, Crohn's disease, diabetic nephropathy and pulmonary hypertension [99-101]. TGF-β1 induces fibroblast differentiation into myofibroblasts resulting in increased deposition of extracellular matrix and collagen type-I, leading to decreased function of the affected organ [102]. Endoglin is overexpressed in liver- and renal fibrosis and scleroderma tissues, indicating a role for endoglin in fibrosis [100]. However, contradictory findings on the pro- or anti-fibrotic role of endoglin in TGF-β-induced fibrosis have been reported (reviewed in [101]).

Recently, Kapur and coworkers performed a study on the role of endoglin in cardiac fibrosis. This group showed that reduced endoglin expression results in better left ventricular function, reduced collagen deposition and increased survival rates in a mouse model of heart failure, compared to mice with wild type endoglin levels [92]. To further explore the

therapeutic potential, these mice were treated with sEndoglin, which acts as a ligand trap for cytokines that normally bind to membrane-localized endoglin (Fig.2). These data showed decreased TGF-β signaling and improved heart function following treatment. In conclusion, endoglin seems to be involved in fibrosis, although its exact function is not known.

Tumors are, in addition to the epithelial tumor cells, composed of cancer-associated fibroblasts (CAFs), which show similarities to the activated fibroblasts observed in fibrotic tissues. TGF-β signaling is known to play an important role in the activation of these CAFs [103, 104]. Moreover, our recent work has shown hyperactivation of TGF-β signaling after interaction with epithelial cancer cells [105]. Based on the similarities between fibroblasts and CAFs and their activation by TGF-β, this opens the interesting possibility of a potential role for endoglin signaling in CAFs during cancer progression. Recently, Vary and colleagues have shown that endoglin expression on CAFs influences tumor growth and metastatic potential of cancer cells in a mouse model of prostate cancer [106]. However, since endoglin was reduced in all cell types, this effect could also be caused by decreased tumor vascularization in endoglin haploinsufficient ($Eng^{+/-}$) mice [106]. These $Eng^{+/-}$ mice develop smaller and less metastatic prostate cancers and these tumors lack CAFs when compared to $Eng^{+/+}$ mice. Yet, this effect was not observed in endoglin haploinsufficient xenografted lung carcinomas [107], suggesting a prostate cancer-specific mechanism. Additional studies will reveal the involvement of endoglin dependent signaling in CAF activation and their contribution to cancer progression and metastasis.

3.2 Cancer

3.2.1 Endoglin and tumor angiogenesis

Anti-angiogenic therapies, mainly targeting the VEGF pathway, have been approved for patients with various solid tumors including renal, lung and gastrointestinal tumors [108]. Anti-angiogenic therapy can be applied both as monotherapy and in combination with conventional chemotherapy. Both approaches have been effective in tumor regression and inhibition of metastasis in preclinical and clinical settings [109, 110]. However, clinical anti-tumor effects have been temporary and are followed by tumor progression. Resistance is caused by normalization of the tumor vasculature (i.e., improved vessel functionality) and the ability of the ECs to overcome anti-angiogenic treatment by upregulating alternative anti-angiogenic pathways [7].

Therefore, targeting other angiogenic pathways, including proteins highly expressed on proliferating vessels, might be a promising approach. Endoglin is such a candidate, as it directs TGF-β signaling towards a proliferative and migratory phenotype, is essential for angiogenesis and is highly overexpressed in proliferating rather than quiescent vasculature. Therapy directed at endoglin is therefore expected to manifest anti-angiogenic effects specifically in the cancer vasculature.

Since endoglin is highly and specifically expressed on tumor ECs, it could also be used as a marker to assess vascularization of a tumor. The number of microvessels per high power

field in the areas of most intensive neovascularization, also called microvessel density (MVD), is a very useful marker for the prognosis of many cancer types [111]. MVD can be assessed using a variety of endothelial cell markers, including CD34, CD31 (PECAM) or von Willebrand factor, all of which have different predictive values [112]. With regard to endoglin, several studies have shown an inverse correlation between endoglin MVD and overall and disease-free survival in breast cancer patients [112-114]. In endometrial cancer, low endoglin MVD was associated with increased overall survival and was shown to be superior to CD34 and VEGF staining [115]. Additionally, endoglin MVD in early stage cervical cancer was the best predictor for the presence of lymph node metastases [116] and was shown to be an independent predictor of survival in ovarian cancer [117]. A similar observation was made for oral squamous cell carcinoma [118]. Endoglin MVD was also related to metastasis, tumor aggressiveness and survival in esophageal, oral and head and neck cancer as well as tumor recurrence and disease-free survival in laryngeal carcinomas [119-122]. In colorectal cancer, endoglin MVD correlated with both lymph node and liver metastases [123] and correlated with the risk of progression from dysplasia to carcinoma in colorectal mucosa [124]. Endoglin MVD was correlated with survival in non-small cell lung cancer [125] and hepatocellular carcinomas [126], and with tumor stage and metastasis in prostate cancer [127] and breast cancer [113]. Recently, Lin and colleagues showed that endoglin MVD is an independent prognostic marker in predicting survival of cervical cancer patients after receiving chemoradiation therapy [128] These studies indicate that endoglin MVD has a higher prognostic value compared to other endothelial markers, including CD34, CD31 and von Willebrand factor.

3.2.2 sEndoglin in cancer

In addition to endoglin MVD, the soluble form of the receptor generated via MMP-14-dependent cleavage of membrane bound endoglin [53], has been evaluated for prognostic purposes. Although ECs are the most probable source, a role for other cells (e.g. tumor cells or fibroblasts) in contributing to sEndoglin levels in the plasma of patients cannot be excluded [129]. Several researchers have reported increased levels of sEndoglin in serum of pregnant women diagnosed with pre-eclampsia [67, 130]. sEndoglin has been correlated with metastatic disease in breast and colorectal cancer patients [131, 132]. However, other groups reported no changes in sEndoglin plasma levels in breast, gastrointestinal and esophageal cancer patients [133-136]. Differences in methods to quantify sEndoglin may explain variations between these studies. In patients suffering from both liver cirrhosis and hepatocellular carcinoma (HCC), sEndoglin serum levels were reported to be about two times higher than in patients suffering from either one of those conditions alone [137]. These data suggest that increased sEndoglin levels could indicate the presence of HCC in cirrhotic patients. sEndoglin levels in the urine of prostate cancer patients were found to be correlated with disease stage, lymph node metastases, tumor aggressiveness and recurrence [138-140]. Taken together, although there are indications that sEndoglin could have some diagnostic or prognostic value,

studies are not conclusive and more studies are required to evaluate the prognostic value of sEndoglin in cancer.

3.2.3 Endoglin as tumor vasculature imaging tool

Non-invasive molecular imaging plays an important role during cancer diagnosis and treatment, and potentially could lead to personalized treatment because of improved tumor characterization [141]. The luminal localization on angiogenic vessels makes endoglin easily accessible by antibodies and therefore renders it an interesting candidate for tumor imaging (Fig. 3A). Among different imaging techniques, ultrasound imaging is commonly used in the clinic because of its safety, low costs and ease of use, but tissue penetration is low. Anti-endoglin antibodies were used to visualize tumors by ultrasound to monitor anti-angiogenic treatment response in mouse models. Avidin-coated microbubbles were used to bind biotinylated MJ7/18 anti-endoglin antibodies. The data revealed highly specific binding to endoglin expressing cells in the tumor vasculature in mice bearing orthotopic human pancreatic tumors [142]. The ultrasound signal intensity of specific endoglin-conjugated microbubbles correlated with the MVD during treatment. Although the sensitivity of ultrasound is limited, improvements are possible through the use of advanced contrast agents and antibody-contrast agent conjugation methods [143].

An imaging technique offering higher contrast, spatial resolution and tissue penetration than ultrasound, is molecular MRI (mMRI). mMRI combines the acquisition of anatomical structures of MRI with the detection of biological processes at the molecular level [144]. In a recent report, the potential use of endoglin as an imaging agent in combination with mMRI was analyzed. Radioactive iron (^{59}Fe) coupled endoglin MJ7/18 antibodies increased the sensitivity of detecting subcutaneous tumors in mice by mMRI, compared to control Gd-coupled antibodies [144].

The most promising methods for tumor imaging are radionuclide-based, including positron emission tomography (PET) and single photon emission computed tomography (SPECT), due to their high sensitivity and tissue penetration [15, 145, 146]. Several animal studies were performed to analyze the potential of endoglin as an imaging tool for the tumor vasculature. One group showed efficient imaging of melanomas upon intravenous injections of ^{111}Indium Chloride-labeled MJ7/18 anti-endoglin antibodies in a mouse model [145]. Autoradiography and immunohistology showed the highest signal at the tumor edges, where vascular density appeared to be highest. Additionally, in a spontaneous breast cancer model in dogs, ^{125}Iodine-labeled MAEND3 anti-human endoglin antibodies were rapidly taken up by the tumors and produced an intense signal without systemic side effects [15]. Furthermore, Cai and coworkers investigated the future clinical applicability of using PET scans to image the tumor vasculature in a mouse model. Injection of graphene oxide-conjugated TRC105 anti-endoglin antibodies resulted in specific imaging of the tumor vasculature in mice by PET imaging [147]. Specific tumor uptake of the antibodies was confirmed by immunohistochemistry. Although promising, improvements are possible

concerning the rate of antibody accumulation in the tumor and the background level in the clearing organs (e.g., the liver).
PET combined with near-infrared fluorescence (NIRF) imaging, known for its high resolution, was also explored for tumor imaging. Zhang and coworkers labeled TRC105 with both a NIRF dye and radioactive copper (^{64}Cu) and demonstrated specific tumor imaging in an *in vivo* breast cancer model using NIRF and PET imaging [148]. Although NIRF has low tissue penetration, it can be very useful during endoscopy and image-guided surgery.
The potential clinical use of endoglin-based imaging in cancer diagnosis was underlined when fresh nephrectomy specimens from patients with renal cell carcinoma were perfused with a ^{99}Technetium-labeled E9 anti-endoglin antibody. The observed radiographic hot spots corresponded with tumors identified in preoperative MRI scans. Remarkably, another tumor was identified, which was not observed in preoperative scans using the labeled anti-endoglin antibody [146].
These data suggest that endoglin-based tumor imaging is promising, although there is still room for improvement before these techniques can be translated to the clinic. Combining different imaging techniques, like PET and NIRF imaging, will likely increase both sensitivity and specificity of endoglin-based tumor imaging and could result in valuable tools for cancer treatment.

4. Endoglin for targeted cancer therapy

Considering the specific overexpression of endoglin on tumor vasculature, it seems to be a promising target for anti-angiogenic therapy. Several approaches targeting endoglin have been evaluated, including endoglin-Fc constructs, endoglin vaccines and endoglin neutralizing antibodies (Fig.3B).

4.1 Endoglin-Fc

Endoglin-Fc is a chimeric protein, consisting of the extracellular part of endoglin fused to the Fc tail of human IgG [44]. This molecule functions as a ligand trap for endoglin ligands including BMP-9 and BMP-10 (Fig. 3B, left panel). Two groups showed that endoglin-Fc inhibits angiogenesis in *in vitro* assays [37, 53] and in an orthotopic breast cancer model (Hawinkels et al., unpublished observations). Furthermore, Castonguay and coworkers showed that endoglin-Fc significantly reduced tumor angiogenesis, and therefore tumor growth, in BALB/c mice subcutaneously inoculated with colon adenocarcinoma cells [37]. Further studies on the therapeutic potential of endoglin-Fc are ongoing.

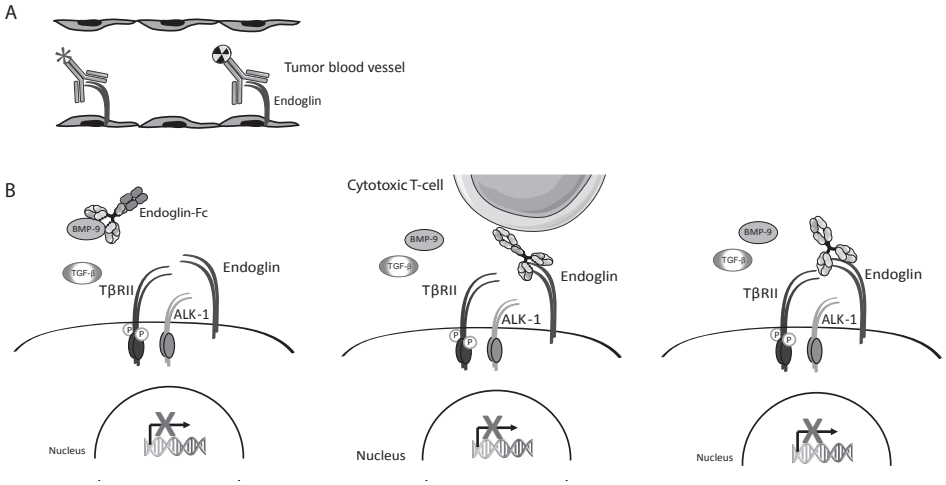


Figure 3 Endoglin antibodies in imaging and targeted treatment. (A) Luminal localization of endoglin on endothelial cells facilitates access of endoglin antibodies. Antibodies labeled with fluorescent (left) or radioactive epitopes (right) can be used in imaging the tumors' vasculature. (B) Left panel; Endoglin-Fc functions as a ligand trap for endoglin ligands, such as BMP-9, thereby inhibiting signaling. Middle panel; Vaccines against endoglin elicit an immune response which results in a cytotoxic T-cell response. Right panel: Endoglin neutralizing antibodies bind to the extracellular domain of endoglin, thereby inhibiting downstream signaling. These strategies result in specific inhibition of tumor-angiogenesis by either inducing cell death (vaccine) or blocking endoglin signaling (endoglin-Fc and neutralizing antibodies).

4.2 Endoglin vaccines

Another possible therapeutic approach in solid cancers might be immunotherapy directed against antigens expressed on the surface of the tumor vascular ECs. Such therapies elicit an immune response against cells expressing this antigen (Fig.3B, middle panel). When the antigen is tumor vessel-specific, such as endoglin, it abrogates tumor angiogenesis [149]. One study explored the use of an endoglin protein vaccine with low dose cisplatin. Lung or liver cancer cells were subcutaneously injected in mice treated with the protein vaccine, cisplatin or a combination [150]. Both monotherapies inhibited tumor growth, while combination therapy synergistically decreased tumor volume, induced auto-antibodies against endoglin and induced EC apoptosis *in vivo* [150]. More recently, endoglin-based DNA vaccines were developed and studied in pre-clinical models. Wood and coworkers reported that treatment with *Listeria monocytogenes* containing an endoglin-expressing plasmid inhibited the growth and metastasis of established breast cancers *in vivo* [151]. In another study, attenuated *Salmonella Typhimurium* containing an endoglin-expressing plasmid was orally administered to mice bearing subcutaneous tumors. Treatment inhibited tumor growth, enhanced T-cell response, inhibited tumor angiogenesis and mediated endoglin-specific cytotoxicity [152]. These effects were enhanced when the treatment was combined with either intratumoral administration of IL-12 or systemic cyclophosphamide treatment. Importantly, treatment did not affect physiological angiogenic processes, such as wound healing.

4.3 Endoglin neutralizing antibodies

In addition to targeting endoglin with the use of endoglin-Fc chimeras or endoglin based vaccines, much effort has been devoted to targeting endoglin with neutralizing antibodies (Fig.3B, right panel). Seon and colleagues showed that targeting the tumor vasculature using deglycosylated ricin A-chain-coupled anti-endoglin antibodies suppressed growth of subcutaneously established human breast cancers in immunodeficient mice [153]. Combination therapy of anti-endoglin an antibody with the chemotherapeutic drug cyclophosphamide produced synergistic antitumor effects [154]. Anti-endoglin antibodies, either unconjugated or conjugated to toxins, also suppressed the formation of metastases from mammary and colorectal carcinomas in mouse models [155].

Tsujie *et al.* investigated the working mechanism by which unconjugated endoglin antibodies inhibit tumor growth and suppress angiogenesis. They observed that the anti-endoglin SN6j antibody was more effective in suppressing tumor growth in immunocompetent mice than in severe combined immunodeficiency (SCID) mice [156]. Depletion of $CD4^+$ and/or $CD8^+$ T-cells abrogated the tumor inhibitory effects of SN6j, indicating that antibody activity is dependent on an immune response. However, SN6j also induced apoptosis in human ECs *in vitro* [156], suggesting an additional mechanism of action, for example loss of inhibition of hypoxia-mediated cell death by endoglin [98].

TRC105 is a chimeric IgG1 monoclonal antibody, based on the SN6j antibody, that binds human endoglin with high affinity and induces antibody-dependent cytotoxicity and apoptosis of endoglin-expressing cells [157]. Recently, results from a first phase-I clinical trial with TRC105 in patients suffering from advanced solid cancers were published [157]. In this phase I trial, 50 patients with advanced solid cancers were included and treated in a dose escalation protocol that explored doses from 0.01 to 15 mg/kg. Nearly half of the refractory cancer patients enrolled in the trial were progression-free after 2 months and 14% were progression-free after 4 months of TRC105 treatment. Two remarkable cases were observed during the trial. One patient with castrate-refractory prostate cancer, diffuse skeletal metastases and increased prostate specific antigen (PSA) demonstrated marked improvement in bone scans and an undetectable PSA two months after initiation of TRC105 treatment. The bone scan improvement and complete PSA response are ongoing for over 4 years (Fig.4). A second patient with metastatic chemotherapy-refractory uterine carcinosarcoma and several lung metastases demonstrated radiographic regression of disease lasting for 18 months. The duration of TRC105 treatment exceeded the total duration of three prior treatments, each of which was discontinued for progressive disease. Adverse effects were manageable and rarely serious and three patients developed HHT-like telangiectases. Overall, TRC105 treatment is well-tolerated at clinically relevant doses that demonstrated anti-tumor activity [157]. Currently, multiple phase-II clinical studies are ongoing (Table 1)[158], evaluating the therapeutic efficacy of TRC105 treatment alone and in combination with bevacizumab and other anti-cancer agents.

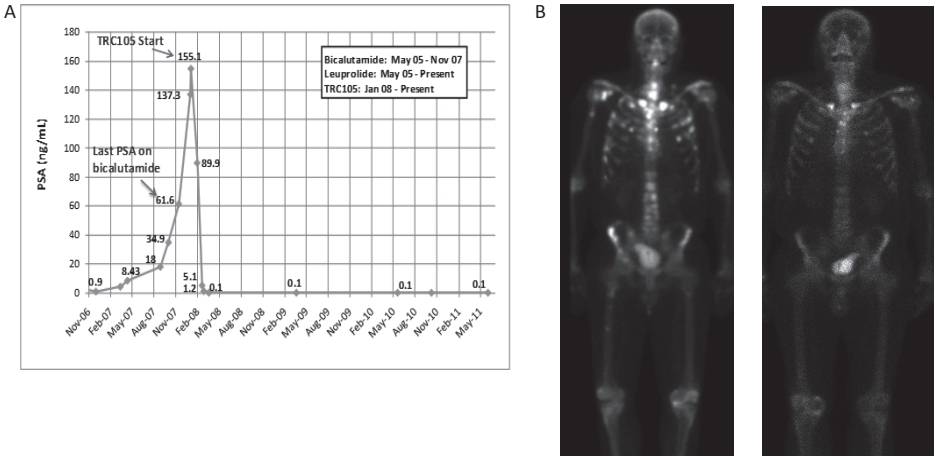


Figure 4 Disease stabilization in prostate cancer after TRC105 treatment. A patient with castrate-refractory prostate cancer showed normalization of PSA levels upon treatment with the anti-endoglin antibody TRC105 (A). Furthermore significant improvement on bone scans (B, right) was observed compared to bone scans before TRC105 treatment (B, left). PSA; prostate-specific antigen. *Reprinted with permission from the American Association for Cancer Research Clinical Cancer Research, 2012; 18 #17; 4820-4829; Rosen et al – figure 2 (A & B) [157].*

5. Conclusion

Because of its selective overexpression and luminal localization on ECs, endoglin is considered to be a promising target for cancer diagnosis and tumor-specific anti-cancer treatment. Endoglin MVD correlates with patient survival and metastatic disease, being superior to several other described markers of angiogenesis. In addition, tumor imaging by endoglin-specific antibody conjugates has been shown to be promising in several pre-clinical *in vivo* studies and awaits clinical translation.

Endoglin targeting for cancer therapy has demonstrated anti-angiogenic responses using either endoglin neutralizing antibodies or endoglin-Fc in several pre-clinical studies. Exciting data were reported from a first-in-human phase-I clinical trial targeting tumor vasculature using anti-endoglin antibodies. Anti-endoglin therapy showed anti-tumor activity at well tolerated doses administered to refractory cancer patients [157]. The results from the ongoing phase II studies, including those of TRC105 in combination with other (anti-angiogenic) therapies will be important to further establish the therapeutic potential of targeting endoglin for cancer therapy.

6. Expert opinion

Anti-angiogenic therapies have been clinically applied for the last decade, but their efficiency in terms of increasing survival of cancer patients has not reached the high expectations indicated by pre-clinical data. Resistance of tumors to therapy and normalization of blood vessels seem to be major problems with the currently approved therapies that primarily target the VEGF pathway. Novel anti-angiogenic therapies are being developed to target unique pathways that may overcome resistance to VEGF therapy, including the TGF-β pathway. Endoglin is a TGF-β co-receptor that is a promising target for tumor imaging and cancer therapy for several reasons:

First, endoglin is highly and specifically expressed on the tumor vasculature, which reduces the risk of side effects. Targeting endoglin will probably induce fewer side effects than targeting other components of the TGF-β signaling pathway, which are expressed in many other cell types and have important roles in tissue homeostasis. Not surprisingly, anti-endoglin antibody treatment was well tolerated in a phase-I clinical trial and demonstrated anti-tumor activity as a single agent [157].

Second, the specific luminal localization of endoglin in vessels opens a window of opportunity to use anti-endoglin therapy for imaging the tumor vasculature. Delivery of antibodies to targets expressed on tumor cells can be hampered by poor tumor penetration. However, endoglin expression on ECs circumvents the necessity of tissue penetration. Labeling anti-endoglin antibodies with radioactive or fluorescent labels could aid in tumor diagnosis and monitor the response during treatment to facilitate personalized medicine. Moreover, the use of a fluorescent labeled anti-endoglin antibodies could aid surgeons during surgery and decrease the risk of incomplete tumor resection.

In addition to imaging, targeting the tumor vasculature with an anti-endoglin antibody conjugated to chemotherapeutic agents, toxins or radioactive compounds could lead to specific and potent targeting of the tumor vasculature. Such therapies, using radioimmunoisotopes (ibritumomab tiuxetan) and immunotoxins (brentuximab vedotin) have been approved for the treatment of non-Hodgkin's lymphoma. Indications of activity by targeting endoglin with TRC105 as monotherapy in a phase-I trial are encouraging and antibody potency could be augmented through conjugation.

Third, most anti-angiogenic therapies targeting a single pathway promote resistance quite rapidly by upregulating alternative angiogenic pathways. While patients responded to TRC105 monotherapy (inhibition of disease progression and reduction in tumor burden) without serious side effects, it is likely that anti-angiogenic treatments will need to be combined to produce more sustainable anti-tumor effects. In this regard, the combination of anti-VEGF therapy with therapy targeting endoglin, a target that is upregulated following anti-VEGF therapy [159] could prove to be effective in the treatment of various solid cancers.

Finally, although ECs very highly express endoglin, several studies have shown endoglin expression on other cell types within the tumor microenvironment. Recently, it was shown that endoglin is important for TGF-β signaling in fibroblasts [92] and that TGF-β is a major player in the transdifferentiation of resident fibroblasts into CAFs [105, 160, 161]. Additionally, endoglin regulates the invasive and migratory potential of CAFs and tumor cells in prostate cancer [106]. Therefore, targeting endoglin on these cells in addition to the endothelium might enhance the anti-tumor effect. Further studies will be required to elucidate the exact role of endoglin on non-ECs and its potential function in tumor cell migration and invasion.

In conclusion, targeting endoglin seems to be a valuable approach for diagnostic purposes and therapy, both as mono anti-angiogenic therapy and in combination with existing anti-angiogenic or chemotherapeutic treatments.

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Conflicts of interest

The authors declare to have no conflicts of interest to disclose.

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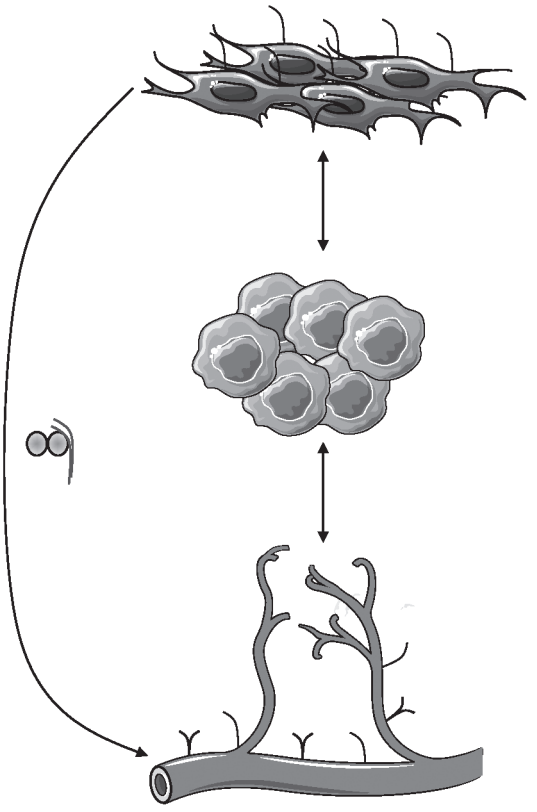
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Chapter 4

Activin Receptor-like Kinase 1 ligand trap reduces microvascular density and improves chemotherapy efficiency to various solid tumors

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Abstract

Purpose: Anti-angiogenic therapy, mostly targeting vascular endothelial growth factor (VEGF), has been applied in cancer patients for the last decade. However, resistance to anti-VEGF therapy and/or no significant benefit as mono-therapeutic agent is often observed. Therefore, new anti-angiogenic strategies are needed. In the present study we investigated the therapeutic effect of interfering with the bone morphogenetic protein (BMP)9/activin receptor-like kinase (ALK)1 signaling pathway by using an ALK1-Fc ligand trap.

Experimental Design: We analyzed the potential anti-angiogenic and anti-tumor effects of ALK1-Fc protein as monotherapy and in combination with chemotherapy *in vivo* in mouse models of melanoma, head and neck cancer and invasive lobular breast carcinomas. ALK1-Fc sequesters BMP9 and 10 and prevents binding of these ligands to endothelial ALK1, which regulates angiogenesis.

Results: Treatment of mice with ALK1-Fc strongly decreased the tumors' microvascular density in the 3 different mouse cancer models. However, this effect was not accompanied by a reduction in tumor volume. An immunohistochemical analysis of the tumor samples revealed that ALK1-Fc treatment increased the pericyte coverage of the remaining tumor vessels and decreased the hypoxia within the tumor. Next, we observed that combining ALK1-Fc with cisplatin inhibited tumor growth in the breast- and head and neck cancer models more efficiently than chemotherapy alone.

Conclusions: The addition of ALK1-Fc to the cisplatin treatment was able to enhance the cytotoxic effect of the chemotherapy. Our results provide strong rationale to explore combined targeting of ALK1 with chemotherapy in a clinical setting, especially in the ongoing phase-II clinical trials with ALK1-Fc.

Introduction

Angiogenesis, the process leading to the development of new blood vessels, is essential for the outgrowth of solid tumors, providing nutrients and a mechanism for dissemination to cancer cells (1, 2). Notably, the newly formed tumor vasculature is structurally and functionally abnormal leading to an aberrant tumor microenvironment characterized by interstitial hypertension, hypoxia, and acidosis. Collectively, these abnormalities contribute to impaired delivery and reduced efficacy of therapeutics to solid tumors.

Anti-angiogenesis therapies aim to prevent the formation of new blood vessels and ultimately starve the cancer cells and inhibit tumor progression. A broad number of molecules have been identified and shown to play key roles in this process (3). Among them, vascular endothelial growth factor (VEGF) has been used as the prime anti-angiogenic target in the clinic for the last decade (4). Unfortunately, in contrast to the promising results from pre-clinical studies, the use of these anti-angiogenic agents as monotherapy has yielded only modest therapeutic benefit in some tumor types, whereas it has failed in others (5-7). Moreover, after an initial response, resistance to this therapy is often observed. In addition, long-term suppression of VEGF signaling gives rise to compensatory mechanisms by other angiogenic pathways (8). Therefore, alternative anti-angiogenic targets should be explored (9).

When combined with chemotherapy, synergistic effects have been reported (10, 11). However, clinical trials of breast cancer patients with the anti-VEGF antibody bevacizumab, also in combination with chemotherapy have shown variable results and no clear clinical improvement yet (12). A major challenge remains that a large number of cancer patients do not respond at all, or only minimally, to anti-angiogenic therapies.

Activin receptor-like kinase (ALK)-1 represents a promising target for anti-angiogenic therapy in solid tumors. ALK1 forms part of a transforming growth factor β (TGF-β) receptor signaling complex on endothelial cells and plays an important role in regulating angiogenesis (13, 14). Bone morphogenetic protein (BMP)9 and BMP10 bind directly to ALK1 (15, 16). ALK1-Fc, a chimeric protein consisting of the ALK1 extracellular domain fused to the Fc-part of an antibody, functions as a ligand trap and sequesters BMP9 and 10 and prevents binding of these ligands to endothelial ALK1 receptor, thereby decreasing angiogenic responses *in vitro* (17). However, the role of BMP9 in angiogenesis remains controversial and seems to be strongly context and concentration dependent (18).

A previous study has shown that administration of a BMP9 neutralizing antibody or ALK1-Fc causes increased retinal vascularization due to elevated BMP10 levels (19). Conversely, ALK1-Fc treatment was shown to inhibit MCF-7 breast cancer growth *in vivo* (20). Interestingly, ALK1-Fc was recently reported to have therapeutic effects in multiple patients with advanced cancer (21). Therefore, identifying what types of tumors might be sensitive to this therapy could yield long-term survival benefits to these patients.

In this study, we analyze the effects of targeting ALK1 ligands with the ALK1 ligand trap ALK1-Fc *in vivo* using three different mouse cancer models of melanoma, head and neck

cancer and invasive lobular breast carcinomas. Mortality rates of patients with melanoma, head and neck cancer and breast cancer have not significantly decreased in past years. Currently, these tumors account for over 20% of the estimated new cases of cancer in 2014 and approximately 10% of cancer-related mortality (data from American Cancer Society), indicating a clinical need for new therapies. Angiogenesis has a well-recognized role in these types of tumors and several studies have shown a correlation between disease progression and vascular density. However, to date, few clinical trials have shown promising results in these types of cancers using anti-angiogenic agents. Therefore, we sought to determine the anti-tumor effects of interfering with the ALK1 signaling pathway by using an ALK1-Fc ligand trap.

Materials and Methods

Antibodies and recombinant proteins

CD31 (PECAM) antibodies were purchased from Santa Cruz biotechnologies (Santa Cruz, CA, USA) and NG2 antibodies from Milipore (Temecula, CA, USA). Anti-human Fc antibodies were purchased from R&D systems (Abington, UK) and rat anti F4/80 antibodies from eBiosciences (Vienna, Austria). To detect hypoxic areas, hypoxia probe® was purchased (Burlington, Massachusetts, USA). ALK1-Fc is a fusion protein comprised of the extracellular domain of human ALK1 fused to the Fc region of IgG and was generously provided by Acceleron Pharma, Cambridge, USA. ALK1-Fc was dissolved in 20 mM Tris-HCl and diluted in 0.9% NaCl. The Fc domain of IgG_1 was used as control (MOPC-21; Bio Express, West Lebanon NH).

Lentiviral vectors were generated by cloning a sequence containing the ALK1, ALK2 or Flt extracellular domain fused to an Fc expression sequence in pLV puromycin resistance containing plasmids.

Cell lines

KEP1-11 mouse breast cancer cells (22) were isolated from spontaneous breast tumors in E-cadherin$^{-/-}$, p53$^{-/-}$ mice and cells were transduced with luciferase. Cells were kept in DMEM/F12 supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. KEP1-11 cells with stable expression of Fc, ALK1-Fc, ALK2-Fc and Flt-Fc were generated by infecting cells with lentiviruses and selection with 5 µg/ml puromycin (Sigma-Aldrich, Darmstadt, Germany). Luciferase expressing human OSC19 head and neck cancer cells were described before (23) and maintained in DMEM containing 10% FCS, penicillin/streptomycin, MEM-NEAA (1%), MEM vitamin mixture (1%), Glutamax (all Sigma-Aldrich, Darmstadt, Germany), 1mM Pyruvate (Thermo Scientific, The Netherlands).

Animal experiments

All animal experiments using breast and head and neck cancer cells were approved by the animal ethics committee of the Leiden University Medical Center, the Netherlands. All animal studies using melanoma cells were approved by the committee on animal welfare committee of Erasmus MC, the Netherlands.

Treatments consisted of 10 mg/kg bodyweight ALK1-Fc (RAP-041, an ALK1 extracellular domain/Fc fusion protein), Fc control protein or DC101 antibody (10 mg/kg) administered by intraperitoneal (IP) injection twice per week. Cisplatin treatment consisted of 3 rounds, 5 mg/kg IP injections at indicated time points. Doxorubicin was administered by intravenous (iv) injection. Hypoxia probe was injected IP 45 minutes before sacrifice. For perfusion studies 50 µg FITC-lectin (Vector labs, Peterborough, UK) was injected intravenously and after 5 minutes the mouse was anesthetized and perfused with PBS.

For the melanoma model, C57Bl6 mice (Harlan, Indianapolis, IN, USA) were used at a weight of 25 grams. Mice were brought under anesthesia and received subcutaneous Temgesic® (Schering-Plough, Amstelveen, Netherlands) injection as pain medication. Mice were housed under standard conditions or at 30°C and 60% humidity for window chamber experiments.

Window mice for intravital microscopy were generated as previously described (24). Briefly, dorsal skinfold window chambers were installed on the back of mice (n=5) and B16BL6 tumors were generated by inserting a small tumor fragment in the exposed fascia and allowed to grow. Eight days after instalment of the windows and transplantation of the tumors treatment with ALK1-Fc was started with an interval of 2 days between injections. Treatment was continued for the remainder of the experiment. Tumor growth and tumor vessel formation was followed over time. For this purpose endothelial nitric oxide synthase (eNOS)-Tag green fluorescent protein (GFP) mice were used constitutively expressing GFP in endothelial cells allowing visualization of vascular bed formation as described elsewhere (25). Images were made with a Zeiss 510 Meta confocal microscope (Carl Zeiss, Göttingen, Germany). GFP in vessels was visualized by an argon laser (488 nm) with fluorescent band pass filters set to 505-550. Mice were positioned on a heated stage and the windows fixed to prevent motion. Acquired images were analyzed using Image J (Wyne Rasban, NIH, USA).

For the efficacy study tumor fragments were implanted subcutaneously in the flank of mice (n=10-13) as previously described and allowed to grow until palpable (average tumor diameter 4 mm). Treatment was started with ALK1-Fc at that time point and continued every three days. Doxorubicin (DXR) (Pharmachemie B.V. Haarlem, The Netherlands) was added to the treatment starting on the third ALK1-Fc administration with the same interval. Treatment was continued for the remainder of the experiment.

For the breast- and head and neck cancer models, 6-8 week old female Balb/c mice (Charles River, Chatillon-sur-Chalarone, France, strain CBy/cBy.Cg-doc1<nu>/J) of 20 grams were used. Mice were brought under anesthesia and received subcutaneous Temgesic® injection as pain medication. 10 x 10^3 luciferase expressing OSC19 cells dissolved in 10 µl PBS were

injected in the tip of the tongue of the mice (26). 3 days after injection mice were imaged as described above. Treatment with ALK1-Fc or Fc was given twice/week (10 mg/kg, IP) and cisplatin was administrated at days 8, 13 and 18. At day 20, mice were sacrificed and tumors and lymph nodes were removed for further analysis. Blood was collected and serum and plasma samples were stored at -80°C until use.

For the breast cancer model mice (6/group, total 12 mice/group) were brought under anesthesia by IP injection of a ketamine/Sedanum/atropin mixture. Next an incision was made and the 4th mammary gland was exposed. 30 x 10^4 luciferase expressing E-cadherin$^{-/-}$/ P53$^{-/-}$ KEP1-11 cells (22) dissolved in 10 µl of 1:1 medium/Matrigel (BD biosciences, Bedford, MA, USA) solution were injected in the 4th mammary gland. The wound was closed and mice received subcutaneous Temgesic® injection as pain medication. After 2 weeks, mice were injected with D-Luciferin (Synchem, Elk Grove Village, IL, USA) (0.2 mg/g bodyweight), brought under anesthesia by isofluorane inhalation (Pharmachemie, Haarlem, the Netherlands) and imaged using the Xenogen, IVIS Lumina® (Perkin-Elmer, Waltham, MA, USA). Furthermore tumor volume was estimated using caliper measurements and calculated using the formula: Tumor volume = (length^2 x broadness)/2. After two weeks, when tumors were palpable, mice were treated twice/week IP with 10 mg/kg ALK1-Fc, or Fc control protein. For cisplatin studies mice were treated once/week IP for 3 weeks with 5 mg/kg cisplatin (Tocris, Bristol, UK). Weight was monitored several times per week. After 6.5 weeks mice were sacrificed, tumors and metastasis were collected and processed for standard immunohistochemistry. Blood was collected and serum and plasma samples were stored at -80^{0}C until use.

Immunohistochemistry

Tumors were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. 4 µm sequential sections were cut and mounted on superfrost glass slides (ThermoScientific, Amsterdam, The Netherlands). Immunohistochemistry was performed as described before (27). In short, slides were incubated overnight with primary antibodies, followed by biotinylated secondary antibodies (all obtained from Dako, Glostrup, Denmark). Staining was visualized by the vectastain system (Vector laboratories, Peterborough, UK) and diaminobezindine staining (Sigma-Aldrich, Darmstadt, Germany). Counterstain was performed with haematoxylin and slides were mounted using entellan (Merck, Darmstadt, Germany). For microvessel density analysis, binary images were generated and staining was quantified using ImageJ software. Four fields per tumor were analyzed to calculate the average microvessel density per mouse.

Immunofluorescence

Immunofluorescence staining was performed as described previously (28). In brief, immunofluorescence staining of tumors samples was performed on 4µm paraffin embedded sections. After deparafination and rehydration antigen retrieval was performed by boiling in 0.01M sodium citrate, pH 6.0, for 10 minutes followed by overnight incubation at 4°C

with NG2 and CD31 antibodies. Next, sections were incubated with secondary antibodies labeled with Alexa Fluor 488, 555, or 647 (Invitrogen/Molecular Probes, Waltham, USA) at 1:250. Nuclei were visualized by DAPI, which was included in the mounting medium, Prolong G (Invitrogen/Molecular Probes, Waltham, USA).

TGF-β1 and BMP9 ELISAs

Plasma BMP9 levels were measured by ELISA as described before (29). TGF-β1 levels were measured using a commercially available mouse TGF-β1 Duoset ELISA (DY1086, R&D systems, Abington, UK) as described before (30, 31).

Western blot analysis

To evaluate expression of the Fc constructs in the stable cell lines, cells were lysed and 10 µg protein was separated by 10% SDS-PAGE. Western blotting with anti-Fc antibodies (1:1000, R&D systems, Abington, UK) was performed as described before (27).

Statistical analysis:

Differences between groups were calculated using the student's t-test or Mann-Whitney U-test when appropriate.

Results

ALK1-Fc reduces tumor vascular density without inhibiting tumor size in melanomas, head and neck cancer and breast cancer.

The pro-angiogenic ALK1 receptor has been shown to bind to BMP9, which is present in circulation (32). Before testing the effect of sequestering these proteins *in vivo* by injecting ALK1-Fc, we investigated the expression of BMP9 in human tumor samples. Immunohistochemical analysis revealed that BMP9 was clearly expressed in the squamous cell tongue carcinoma and invasive lobular breast carcinoma samples tested (supplementary Figure 1). To determine the effect of ALK1-Fc protein on vascularization and tumor development, OSC19 human oral squamous carcinoma cells were injected in the tip of the tongue of Balb/c nude mice. These cells carry a luciferase construct, allowing us to follow tumor growth and development in time by bioluminescence imaging (BLI) (Figure 1A). Size of these fast-developing tumors was determined by quantifying the luciferase signal after administration of luciferin (Figure 1B). Initial imaging 4 days after injection was performed to randomize the mice in groups with equivalent signal and thereafter mice were treated systemically with the anti-angiogenic agent. The impact of ALK1-Fc was compared to Fc control and DC101, a well-known anti-mouse VEGF receptor (VEGFR) antibody (33). Treatment with ALK1-Fc did not affect tumor growth compared to the control group (Fc). As expected, tumor growth inhibition was observed in mice treated with DC101, resulting in a significantly decreased tumor size at day 20 (Figure 1A and 1B).

At this point, tumors were retrieved and immunohistochemical analysis was performed. Staining with CD31 specific antibodies, a marker for endothelial cells, revealed that treatment with either ALK1-Fc or DC101 led to significantly reduced vascular density in the tumors (Figure 1C, representative images are shown in supplementary Figure 2A). These results suggest that interference with ALK1 or VEGF signaling inhibits tumor angiogenesis, but only the latter affects growth of oral squamous cell tumors.

To assess the therapeutic effects of ALK1-Fc in melanoma, a syngeneic B16 melanoma model was used and the development of the tumor and vasculature was followed using intravital microscopy in a dorsal skinfold window chamber. Administration of ALK1-Fc did not affect tumor growth (Figure 1D), but had a clear effect on vascular density of the melanomas (Figure 1E and 1F). In the Fc treated mice, 34% GFP positive pixels (reflecting vessel quantity) were observed, whereas in the ALK1-Fc treated mice this was reduced to 19%. This corresponds to a reduction of about 45% of the vascular density (Figure 1E and 1F). Next, the potential therapeutic effect of ALK1-Fc treatment on the growth and vascularization of tumors was tested on a model for invasive lobular breast carcinomas (22). Mice were injected orthotopically with the luciferase-expressing KEP1-11 breast cancer cells. After 2 weeks, when tumors were palpable, mice were treated with PBS, Fc, ALK1-Fc or DC101. Our data show that tumor size, as monitored by BLI (Figure 2A) and weight of the tumors *ex vivo* was not affected by treatment with ALK1-Fc, whereas it was reduced after treatment with DC101 (Figure 2B). Immunohistochemical detection of endothelial cells using a CD31 specific antibody revealed strongly reduced vascular density in both the ALK1-Fc treated and the DC101 treated tumors compared to the Fc control tumors (Figures 2C and 2D). Since tumor infiltrating macrophages have been shown to have important roles in the regulation of tumor angiogenesis, we stained for F4/80 to determine macrophage accumulation in the tumors. Our data show no significant difference in macrophage accumulation in ALK1-Fc treated mice versus Fc control mice (supplementary Figure 2B).

To analyze if ALK1-Fc could interfere with tumor initiation, we generated KEP1-11 breast cancer cell lines expressing ALK1-Fc, ALK2-Fc (ALK2 is another BMP type-I receptor), Fc (negative control) and Flt-Fc (soluble VEGF-receptor, positive control). Expression of the constructs was verified using western blot analysis on tumor cell lysates (supplementary Figure 3A). After orthotopic transplantation of the cells, tumor development was followed by BLI and tumor growth was assessed by quantification of the luciferase signal and caliper measurements. ALK1-Fc expression reduced circulating BMP9 levels in mice to undetectable levels (supplementary Figure 3B), whereas tumor tissue- and circulating-TGF-β1 levels were not affected (data not shown). Our data show that tumor growth was not affected by expression of ALK1-Fc or ALK2-Fc compared to the Fc control (Figure 2E and 2F). Only Flt-Fc expression on these cells led to a significant reduction in tumor size (p=0.03). Immunohistochemical

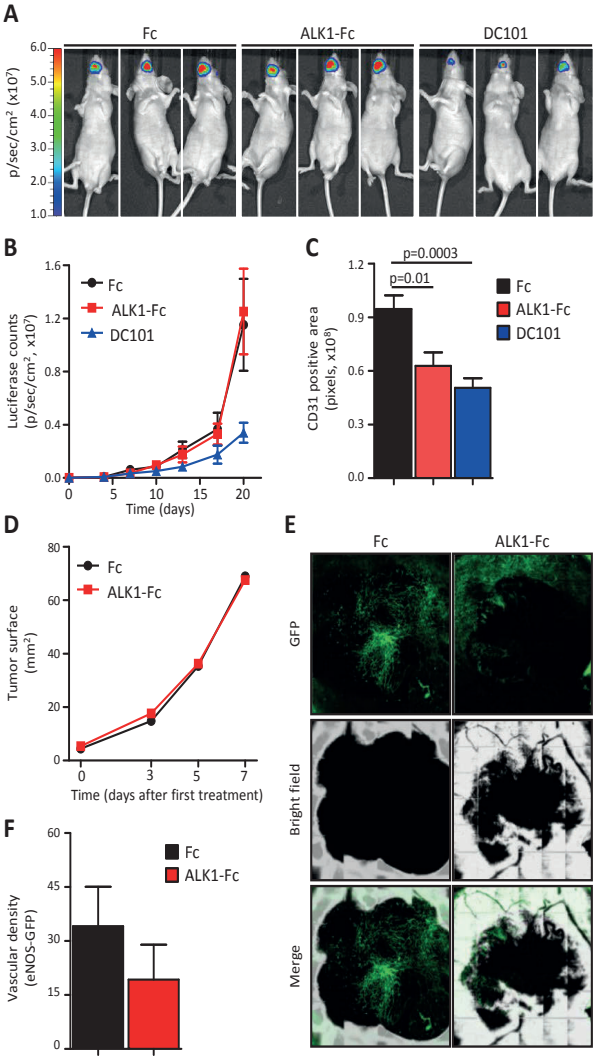


Figure 1. Effect of ALK1-Fc treatment on tumor growth and vascularity in head and neck cancer and melanoma mouse models. A, Bioluminescence images of Balb/C nude mice injected with OSC-19 luciferase expressing cells in the tongue (day 20). Mice were treated with Fc, ALK1-Fc or DC101 (n=8 per group) and pictures from 3 representative mice per group are shown. B, Tumor growth was assessed by quantification of the luciferase signal on days 4, 7, 10, 13, 17 and 20. C, The anti-angiogenic effect of the treatments was verified by immunohistochemical analysis of the tumor samples and quantification of the CD31 positive area. D, Dorsal skinfold window chambers were installed on the back of C57/Bl6 mice and a small tumor piece from subcutaneous B16 tumors were inserted in the exposed fascia. After 8 days, treatment with either ALK1-Fc or control Fc started and tumor development was assessed by measuring the tumor surface on days 0, 3, 5 and 7 (n=2). E, To visualize the vascularization of the tumors, eNOS-GFP mice were used which constitutively express GFP in endothelial cells. Representative images from the window chambers show the vascularization of the tumors upon treatment with Fc or ALK1-Fc (n=5 per group). F, Quantification of vascular density in Fc and ALK1-Fc treated melanomas (day 13).

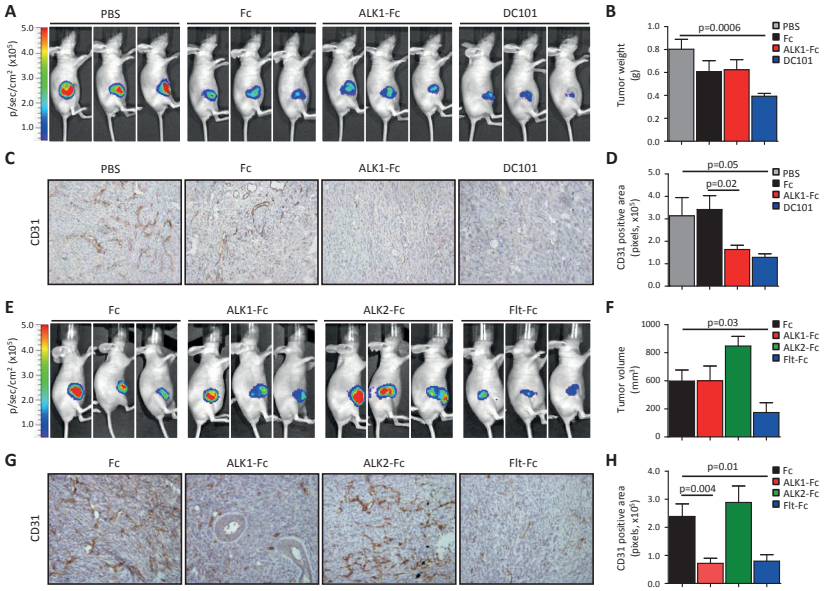


Figure 2. Effect of ALK1-Fc on tumor growth and vascular density in breast cancer mouse models. A, Bioluminescence images of nude mice orthotopically injected with KEP1-11 luciferase expressing cells in the fourth mammary gland. Mice were treated with PBS, Fc, ALK1-Fc or DC101 (n=8 per group) and pictures from 3 representative mice per group are shown (week 7). B, Mice were sacrificed and tumors were retrieved and weighted. Data are presented as mean ± SEM. C, Representative pictures of CD31 staining on the tumors from each group are shown (Original magnification 100X). D, Quantification of the CD31 positive area. E, Breast cancer KEP1-11 luciferase expressing cells with stable expression of Fc, ALK1-Fc, ALK2-Fc and Flt-Fc were orthotopically injected in nude mice and tumor burden and growth was followed by BLI. Representative pictures from 3 mice per group are shown. F, Tumor growth was assessed by caliper measurement and quantification of the tumor volume at end stage is shown. Graph represents mean ± SEM. G, Representative pictures of immunohistochemical analysis of vascularization in tumors using CD31 specific antibody (original magnification 100X). H, Quantification of CD31 positive area.

analysis of the tumors showed decreased CD31 expression in ALK1-Fc and Flt-Fc expressing tumors compared to Fc control tumors (Figures 2G and 2H).

Together, these data show that although treatment with ALK1-Fc did not result in decreased tumor size, it lead to a strong reduction of the vascular density *in vivo* in three different mouse models for melanomas, head and neck cancer and invasive lobular breast carcinoma.

ALK1-Fc treatment leads to increased coverage of tumor vasculature by NG2 expressing cells.

To investigate why a strong reduction in vascular density does not result in tumor growth inhibition, KEP1-11 mouse breast tumor samples were stained with NG2-specific antibodies, a marker for pericytes. The immunohistochemical analysis revealed increased NG2 staining in ALK1-Fc treated mice (Figures 3A and 3B) or mice bearing ALK1-Fc expressing tumors

(supplementary Figure 4A and 4B). Interestingly, compared to controls the number of pericytes was not increased in tumors from DC101 treated mice (Figures 3A and 3B) or tumors formed after inoculation of Flt-Fc expressing cells (supplementary Figure 4A and 4B).

We next performed immunofluorescent stainings to visualize the localization of the NG2 positive pericytes within the tumors. Mice treated with ALK1-Fc contained CD31 positive cells in the tumor vessels tightly covered by NG2 expressing cells. Endothelial cells forming vessel-like structures in the other treatment groups (PBS, Fc and DC101) were found poorly covered by NG2 positive cells (Figure 3C).

To functionally address the effect of the aforementioned changes on the tumor vasculature, we injected mice with a probe that binds specifically to hypoxic areas. ALK1-Fc treated tumors showed a reduction in the intratumoral hypoxia compared to tumors from control groups (Figure 3D and E). Mice were injected with FITC-lectin prior to sacrifice, and FITC signal relative to CD31 immunofluorescent signal was quantified and calculated for KEP1-11 tumor samples to confirm increased perfusion in ALK1-Fc treated mice versus Fc treated controls (Figure 3F).

Taken together, these results show that ALK1-Fc treatment leads to remodeling of the tumor vasculature with increased pericyte coverage. In addition, we observed increased perfusion and reduced intratumoral hypoxia in mice treated with ALK1-Fc.

ALK1-Fc increases chemotherapy efficiency in melanoma, head and neck cancer and breast tumors.

Considering our previous results, we hypothesized that pretreatment with ALK1-Fc could lead to a normalized phenotype of the tumor vasculature with a temporary improvement of the tumor perfusion that could result in a better delivery and efficacy of chemotherapy in tumors. Therefore, we tested if the co-administration of ALK1-Fc together with a chemotherapeutic drug could increase its cytotoxic effect.

First, the combinatorial effects were evaluated in the KEP1-11 breast cancer model. KEP1-11 cells showed a dose-dependent cisplatin-induced growth arrest (supplementary Figure 5A and 5B). Next, KEP1-11 cells expressing the different Fc constructs were injected in the mammary gland of Balb/c nude mice. After 4 weeks, mice were treated with 3 rounds of cisplatin. In all mice treated with cisplatin, tumor growth was inhibited when compared to the untreated control group. In tumors originating from ALK2-Fc and Flt-Fc expressing cells cisplatin caused a growth inhibition that was comparable to the control Fc tumors, whereas ALK1-Fc expressing tumors showed a further reduction of tumor growth. Notably, complete stasis of ALK1-Fc expressing tumors was observed from the start of cisplatin treatment until the end of the experiment (Figure 4A and 4B).

To obtain further confirmation of the increased cytotoxic effect of cisplatin in combination with ALK1 targeting in a clinically more relevant setting, mice were injected with the KEP1-11 breast cancer cells. Once palpable tumors were formed (2 weeks after injection), treatment with PBS, Fc or ALK1-Fc was started. After 2 additional weeks the

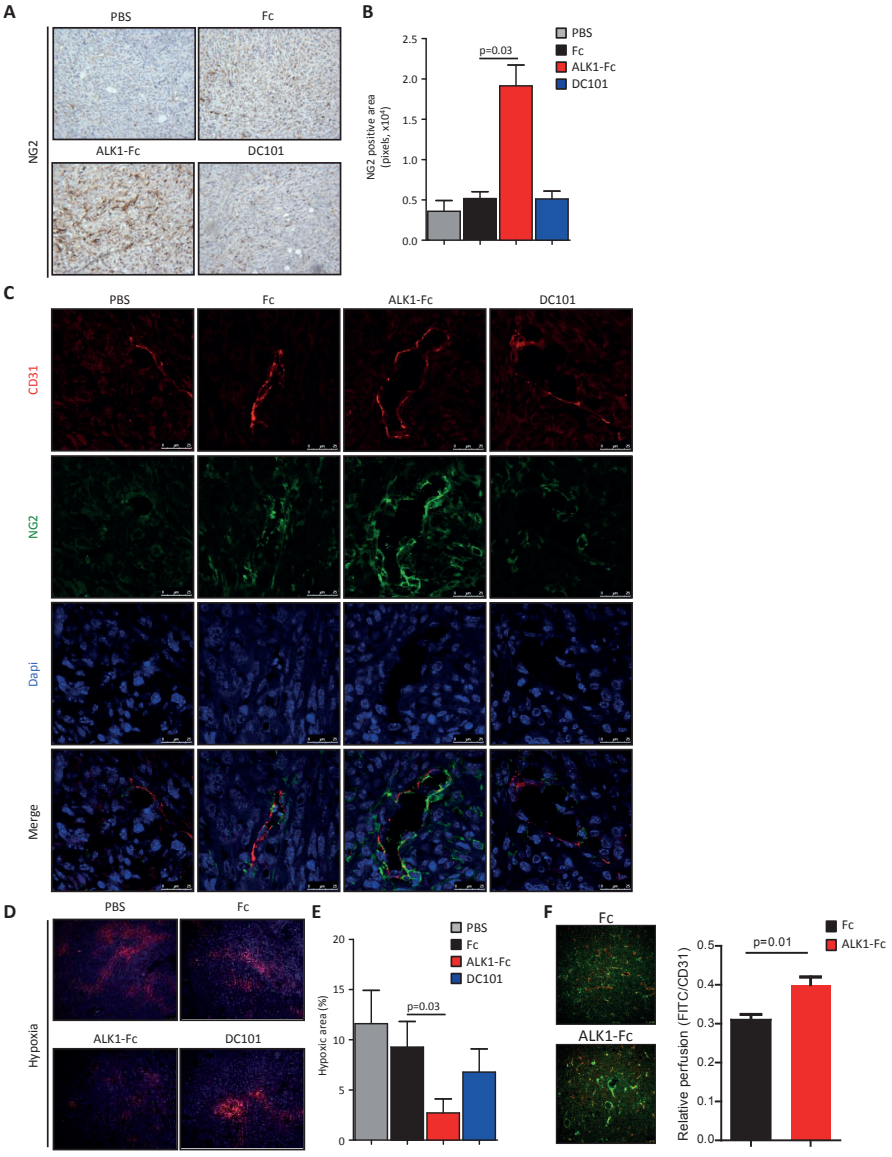


Figure 3. Effect of ALK1-Fc treatment on vessel normalization. A, Changes in the number of pericytes in breast tumors after PBS, Fc, ALK1-Fc or DC101 treatment (n=10-12 per group). Immunohistochemical analysis was performed using NG2 specific antibody (Original magnification 100X). B, Quantification of NG2 positive area. Data are presented as mean ± SEM. C, Representative images of CD31 (red), NG2 (green), Dapi (blue) immunofluorescence staining in KEP1-11 breast tumors treated with PBS, Fc, ALK1-Fc or DC101. Lower row shows merged images. D, Representative images of hypoxia immunofluorescent staining (red) in breast tumors originated after orthotopical injection of KEP1-11 cells. Mice were treated with PBS, Fc, ALK1-Fc or DC101. At end stage (week 7), hypoxia probe was injected prior to sacrifice and was detected by staining the tumor samples with a specific fluorescent antibody. E, Quantification of hypoxic positive area in the tumor samples. F, Quantification of FITC-lectin perfusion in KEP1-11 tumors treated with Fc or ALK1-Fc. Data are presented as mean ± standard deviation, n = 2 (Fc) or 3 (ALK1-Fc) mice, p=0.01.

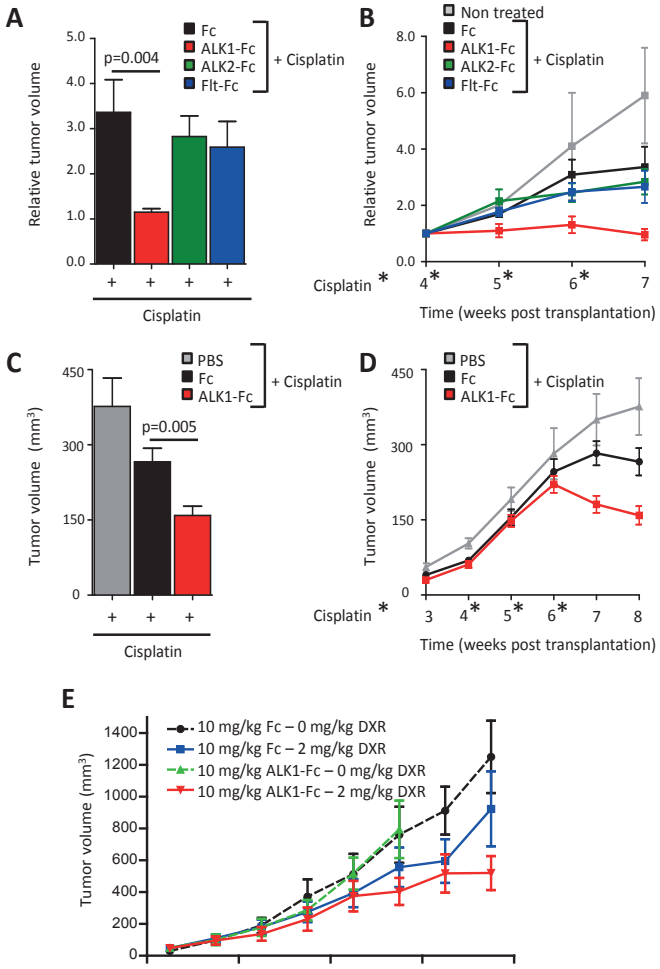


Figure 4. Inhibitory effect of chemotherapy on tumor growth is enhanced when combined with ALK1-Fc. A, Effect of cisplatin treatment on growth of tumors originated from orthotopic injection of KEP1-11 breast cancer cells stably expressing Fc, ALK1-Fc, ALK2-Fc or Flt-Fc constructs. Relative tumor volume at week 7 is shown compared to the tumor volume prior to the start of the cytotoxic treatment. B, Tumor volume was assessed during the duration of the cisplatin treatment. Data are represented as mean ± SEM of the relative volume of tumors compared to the starting point (prior to first administration of chemotherapy) (n=12 mice per group). Asterisks (*) indicate time when cisplatin was administrated. C, Tumor volume of breast tumors originated from orthotopic injection of KEP1-11 in nude mice after 8 weeks. Mice were treated with PBS, Fc or ALK1-Fc in combination with cisplatin. D, Growth and development of tumors was measured during the duration of the chemotherapy. Asterisks (*) indicate time when cisplatin was administrated. Data shown are mean ± SEM. E, Tumor volume of B16 melanomas treated with Fc or ALK1-Fc alone or in combination with the cytotoxic agent doxorubicin (DXR).

therapy was combined with 3 rounds of cisplatin. Consistently with our previous results, administration of ALK1-Fc significantly impaired tumor growth when combined with cisplatin treatment. Furthermore, sustained tumor regression was observed in the last

phase of the experiment, during the 2-week period after the last round of chemotherapy. At the end of the experiment, tumor volume in ALK1-Fc treated groups was significantly smaller, compared to the other groups (PBS and Fc), and similar to the size at week 5, just after initiation of cisplatin treatment. Taken together, these data suggest that pre-treatment with ALK1-Fc renders KEP1-11 tumors more sensitive to chemotherapeutic agents, and represses further tumor development when combined with cisplatin (Figure 4C and 4D).

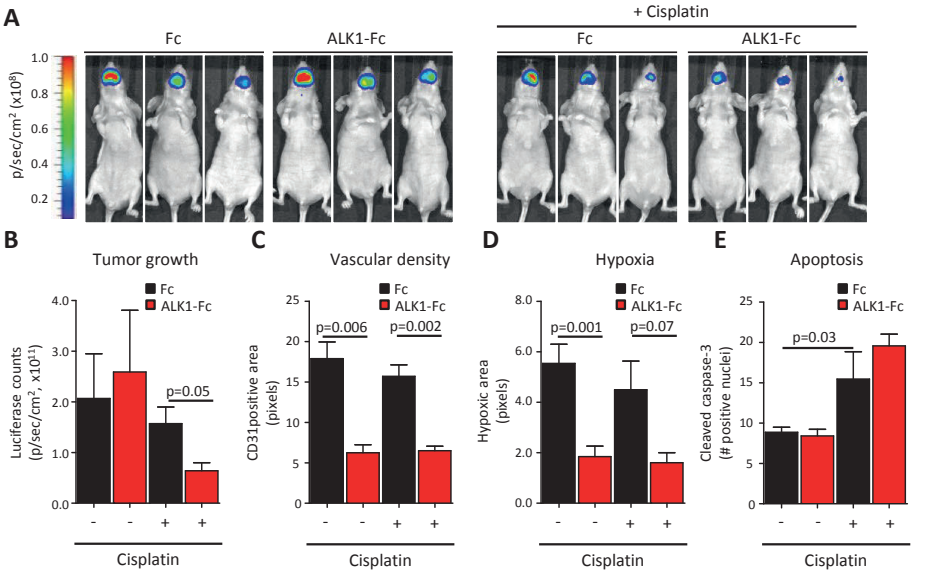


Figure 5. Combination of ALK1-Fc with cisplatin in a head and neck cancer mouse model leads to decreased tumor size by enhancing the cytotoxic effect of chemotherapy. A, Growth of tumors originated from OSC19 luciferase expressing cells injected in the tip of the tongue of nude mice was followed by BLI. Representative bioluminescence images from 3 mice per group are shown at end stage (day 20). Mice received Fc or ALK1-Fc alone, or in combination with cisplatin. B, Tumor size was assessed by quantification of the luciferase signal. Data represents mean ± SEM. C, Tongue tumor samples were embedded in paraffin and stained with CD31 specific antibody for endothelial cells. Vascular density (CD31 positive area) from all the tumors was quantified from the images taken from these samples. Data represented are mean ± SEM. D, At day 20, hypoxia probe was injected in the mice prior to sacrifice and was detected by staining the tumor samples with a specific fluorescent antibody. Quantification of hypoxic positive area in the tumor samples is shown. E, Quantification of cleaved caspase-3 positive nuclei (apoptotic cells) in OSC19 tumor samples.

We next analyzed the response of B16 melanomas, implanted in the back of C57BL/6 mice, to Fc or ALK1-Fc alone or in combination with Doxorubicin (DXR). Mice received two rounds of either ALK1-Fc or Fc prior to the initiation of the chemotherapy. Starting from day 8, 3 cycles of DXR (2 mg/kg) were administered together with Fc or ALK1-Fc in the designated groups. ALK1-Fc alone had no effect on the tumor growth (Figure 4E). DXR exhibited a mild effect on these fast-growing tumors, which was enhanced by co-administration of ALK1-Fc (Figure 4E). In the combination treatment with ALK1-Fc, inhibition of melanoma growth was observed a few days after the first administration of the DXR. This growth inhibition

continued until the end of the experiment, suggesting that pre-treatment with ALK1-Fc leads to increased delivery and/or efficacy of chemotherapy in melanomas.
Finally, ALK1-Fc combination therapy was tested in the human OSC19 head and neck cancer model. OSC19 cells were injected and mice were treated with ALK1-Fc alone or combined with cisplatin. BLI revealed a slight effect of cisplatin alone, which was significantly improved by ALK1-Fc (Figure 5A and 5B). Immunohistochemical analysis showed, that only in the ALK1-Fc groups CD31 staining and hypoxia were decreased (Figure 5C and 5D). While ALK1-Fc did not affect apoptosis when given as a single agent, it did increase the cytotoxic effect of cisplatin (Figure 5E).
Taken together, our data show that combined treatment of ALK1-Fc together with either Doxorubicin or cisplatin enhances the efficiency of the chemotherapeutics in B16 melanomas, OSC19 head and neck cancers and the KEP1-11 mammary tumors.

Discussion

In this study we found that administration of ALK1-Fc can strongly decrease tumor angiogenesis *in vivo*. Treatment with ALK1-Fc significantly reduced the number of vessels in mouse models for melanoma, breast cancer and head and neck cancer. However, despite this effect, tumor size remained unaltered. Further analysis showed that the remaining vessels present in the ALK1-Fc treated tumors exhibited increased number of pericytes, while they were absent or loosely associated with the endothelial cells in the control groups. This change in the tumor vasculature was accompanied by a reduction in hypoxia and an enhancement of tumor perfusion. Combination of ALK1-Fc with chemotherapy further increased the anti-tumoral effect of the cytotoxic agent leading to a reduction in tumor growth in B16, KEP1-11 and OSC19 cancer models. ALK1-Fc was able to increase the cisplatin-induced apoptosis of OSC19 tumor cells. ALK1-Fc treatment may have a therapeutic benefit in patients receiving chemotherapy, either by increasing efficacy or by decreasing chemotherapy dosing regimens and thereby reducing harmful side effects.
The initial aim of anti-angiogenic therapies in cancer patients is to prevent the new formation of vessels within the tumor and impair tumor growth by depriving cancer cells from their increasing demand of nutrients and oxygen. However, in addition to this, by restoring the balance between pro- and anti-angiogenic signaling, anti-angiogenic agents can induce a remodeling of the tumor vasculature, and potentially lead to a temporary improvement in tumor perfusion and reoxygenation. This idea is supported by the "normalization window" concept proposed by Rakesh Jain and colleagues, in which tumor vasculature acquires a normalized phenotype only during a short period of time and depending on the dose of the anti-angiogenic drug used (34).
In line with this, our results showed that treatment with ALK1-Fc efficiently inhibited angiogenesis and reduced the number of vessels present in the tumors, but increased the number of NG2 positive pericytes, which are important regulators of angiogenesis and

vascular stability. In support of the notion that ALK1-Fc may induce a "mature" phenotype of the vasculature we observed reduced hypoxia and increased perfusion in the tumors upon treatment with ALK1-Fc. The mechanism by which ALK1-Fc inhibits angiogenesis is probably by sequestering BMP9 binding to ALK1. BMP9 has biphasic effects on endothelial cells. High doses can inhibit endothelial proliferation and migration (16), whereas low doses can have stimulatory effects (35). A previous study also showed that targeting ALK1 in a different manner by using neutralizing antibodies increases blood flow in larger tumor vessels (36). An alternative explanation for the observed effect of ALK1-Fc on these tumors could include altering the infiltrating inflammatory cells in the tumor mass. However, the number of tumor infiltrating macrophages was not affected in Fc versus ALK1-Fc treated mice.

Our present study corroborated the data obtained in a previous study, in which ALK1-Fc was shown to effectively inhibit angiogenesis *in vitro* (17). In that study ALK1-Fc administration *in vivo* using a model of pancreatic insulinomas could also strongly increase pericyte coverage. However, in this case ALK1-Fc also promoted apoptosis of tumor cells and tumor shrinkage, a phenomenon that we have not observed in our current study. Surprisingly, in all three models used in the current study, strong effects on the tumor vasculature, but not on tumor growth were observed. Treatment of mice with ALK1-Fc alone did not increase apoptosis of cancer cells. Only when combined with chemotherapy a further enhancement of the cytotoxic effect of cisplatin was seen. This effect could indeed be mediated by an enhanced delivery of the drug to the tumor and/or increased cisplatin sensitivity due to reduced hypoxia.

Noteworthy, our data contradicted the results from the first clinical trial using ALK1-Fc that yielded positive effects in head and neck cancer patients (21). The discrepancy between our results obtained in this experimental mouse model and the human clinical trial might be due to the rapid growth of the tumors and/or the lack of an intact immune system in this xenograft model, which could be contributing to the beneficial effect of ALK1-Fc observed in these patients. The temporary enhancement of perfusion could not only lead to increased delivery and efficiency of chemotherapeutic agents, but also to improve the access of the host immune system to the tumors.

Previous studies have shown a correlation between an increase in hypoxia in tumors and the development of metastasis in various models. Since ALK1-Fc seems to decrease hypoxia in these tumors, this could be an additional benefit of the treatment. Unfortunately, the tumor models that we have used do not allow us to look at metastasis formation, since spontaneous metastases do not occur at high frequency. A recently published study on ALK1-Fc in breast cancer however indeed showed an effect on metastasis formation in a breast cancer model (37).

For breast cancer many subtypes are known and also the angiogenic factors playing a role in the induction of tumor angiogenesis seem to be dependent on the histological subtype. The differences in tumor subtype was also highlighted in a recently published paper where the authors could show a reduction in tumor volume in breast cancer after treatment

with ALK1-Fc in a different type of breast cancer (pyMT genetically engineered mouse breast cancer model) (37). Another study showed that invasive lobular carcinomas have a similar microvascular density compared to invasive ductal breast carcinomas. In the latter, expression of VEGF mRNA and protein levels were found to be higher than in the lobular types and correlated significantly with the microvascular density of these tumors (38). This observation suggests that in invasive lobular breast carcinomas, other angiogenic factors besides VEGF might play a significant role. Indeed, we could show strongly decreased angiogenesis in the model for invasive lobular carcinomas by targeting BMP9.

Other approaches to inhibit ALK1 signaling are via small molecules or neutralizing antibodies (39, 40). One of these antibodies, PF-03446962, currently under phase-II clinical development, inhibits binding of BMP9 to ALK1 and prevents BMP9-induced recruitment of the co-receptor endoglin into the signaling complex (41, 42). This antibody has been shown to inhibit tumor growth and could overcome resistance to anti-VEGF therapy (36). Although we could previously show that TGF-β can bind to ALK1 in endothelial cells, it can only do so in the presence of TGF-β type-II receptor (43). TGF-β does not bind to ALK1 alone when expressed on cells (15) or *in vitro* using purified TGF-β and ALK1-Fc proteins in a Biacore analysis. Therefore we do not expect that ALK1-Fc has an effect on TGF-β signaling. Consistent with this notion, we found that ALK1-Fc does not affect TGF-β-induced Smad1 or TGF-β-induced Smad2 phosphorylation, whereas ALK1-Fc does block BMP9-induced Smad1 phosphorylation (17).

Currently, multiple phase-II clinical trials are ongoing for the treatment of solid tumors with ALK1-Fc (dalantarcept) (NCT01720173, NCT01642082), also in combination with sorafinib (NCT02024087) or axitinib (NCT01727336) (44). Interestingly, a phase-II clinical study on the effect of ALK1-Fc on head and neck squamous cell carcinomas has been initiated (NCT01458392). Our current findings indicate that the combination of ALK1-Fc with chemotherapy might be very effective, especially since positive effects of ALK1-Fc treatment (as monotherapy) in head and neck cancer patients have been reported (21). Furthermore, it would be very interesting to test the effect of ALK1-Fc combination treatment in breast cancer patients, where current anti-angiogenic therapies do not show strong therapeutic effects in terms of survival benefit.

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Disclosure of Potential Conflicts of Interests

The authors declare no conflicts of interest.

Translational statement

ALK1-Fc (Dalantercept) is a ligand trap targeting the activin receptor-like kinase-1 and is currently being tested in clinical trials for anti-angiogenic therapy. ALK1-Fc prevents binding of selective bone morphogenetic proteins to endothelial ALK1, which controls (tumor)-angiogenesis. In the current study we show strongly enhanced efficiency of chemotherapy, when combined with ALK1-Fc treatment in various mouse models for solid tumors. These results provide a strong rationale to explore combined targeting of ALK1 with chemotherapy in a clinical setting, especially in the ongoing phase-II clinical trials with ALK1-Fc.

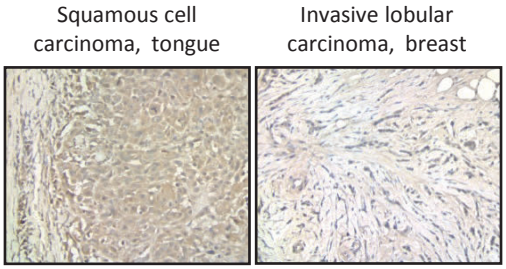
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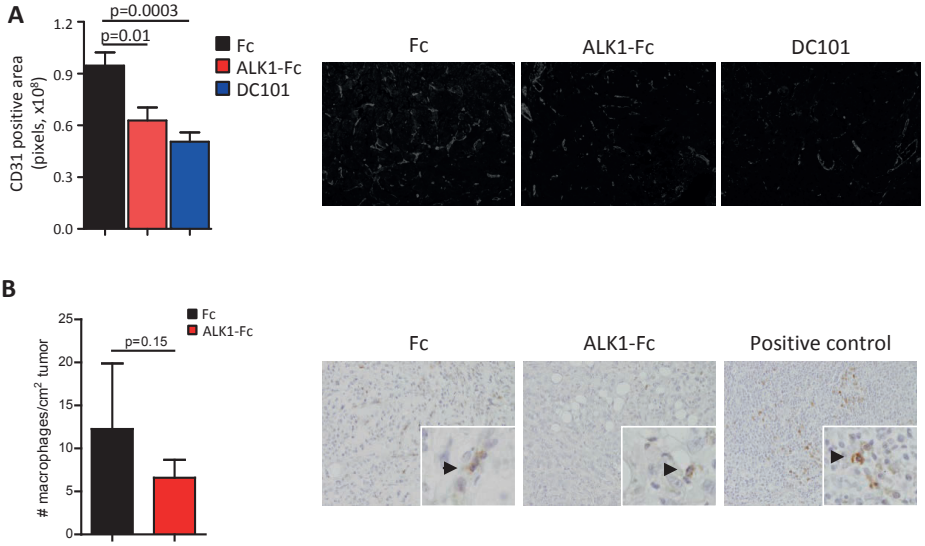
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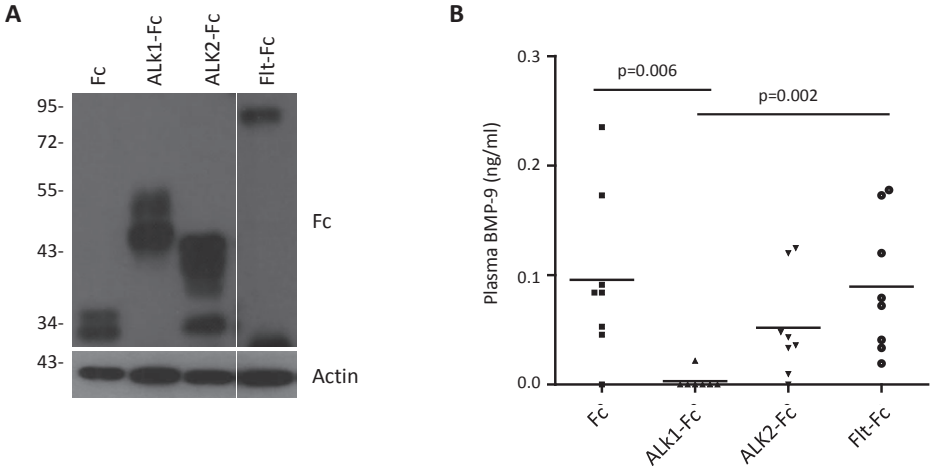
Supplementary data



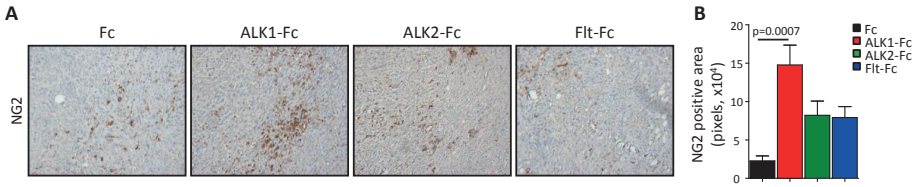
Supplementary figure 1. Immunohistochemical analysis of BMP9 expression in human tumor samples. Pictures are shown from a squamous cell carcinoma of the tongue (left panel), and an invasive lobular breast carcinoma (right panel).



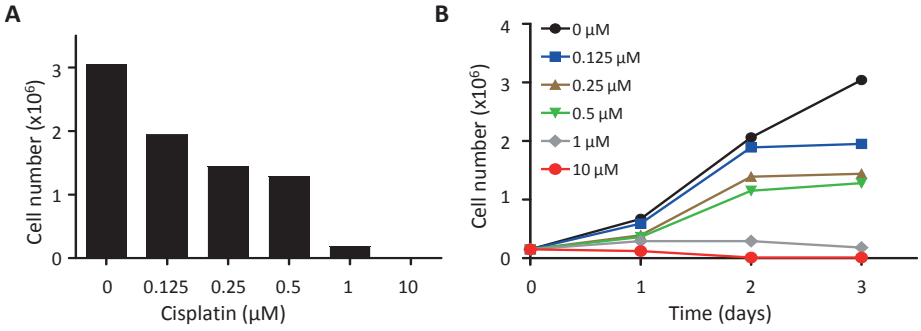
Supplementary figure 2. A CD31 staining in head and neck model. Images taken from tumors after treatment with Fc or ALK1-Fc, magnification 200x. B Images from KEP1-11 tumors stained for macrophages by F4-80, magnification 200x. Total number of macrophages was calculated per cm^2 tumor (n=4 (Fc), n=5 (ALK1-Fc). Positive control: a mouse lymph node showing multiple macrophages.



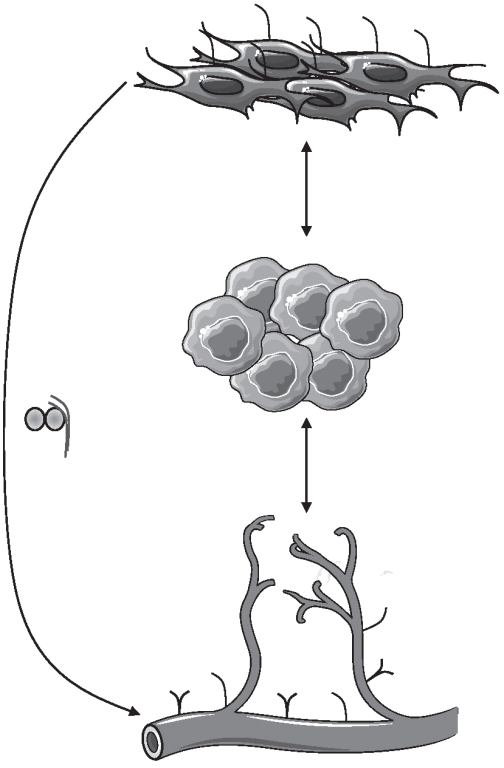
Supplementary figure 3. A, Western blot showing expression of Fc constructs on stable overexpressing cells. B, BMP9 protein levels were measured by ELISA in plasma obtained from mice carrying tumors originated after injection of KEP11-1 cells stably expressing Fc, ALK1-Fc, ALK2-Fc or Flt-Fc constructs.



Supplementary figure 4. A, Changes in the number of pericytes in breast tumors generated after inoculation of KEP1-11 cells carrying expression constructs for Fc, ALK1-Fc, ALK2-Fc or Flt-Fc. Immunohistochemical analysis was performed using NG2 specific antibody (Original magnification 100X). B, Quantification of NG2 positive area. Data are presented as mean ± SEM.



Supplementary figure 5. A, Cytotoxic effect of cisplatin treatment on KEP1-11 cells. Cells were incubated with increasing doses of cisplatin and the number of viable cells was assessed after 72 hours of treatment. B, Dose/response analysis of the effect of cisplatin treatment in time.



Chapter 5

Endoglin targeting inhibits tumor angiogenesis and metastatic spread in breast cancer

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Abstract

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 anti-angiogenic 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*.

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 anti-angiogenic 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, anti-angiogenic 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 anti-angiogenic 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 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 due to 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 anti-angiogenic 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 pro-angiogenic signaling pathways (7), for example the endoglin/transforming growth factor (TGF)-β pathway.

Endoglin is a co-receptor for TGF-β and bone morphogenetic protein (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 anti-angiogenic 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 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.

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 neo-adjuvant setting with bevacizumab. Samples were pre-surgery tumor biopsies (N=5) and tumor tissue obtained after surgery (N=3). Additionally, 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 before (22) using antibodies against CD31 (Santa Cruz Biotechnologies, USA), endoglin (R&D Systems, Abington, UK) or phosphorylated Smad1 (Cell Signaling Technologies, USA). Four representative fields per tumor slide were photographed with a Leitz Diaplan microscope (Leitz, Wetzlar, Germany) and staining was quantified using ImageJ software. ELISA analysis for mouse VEGF was performed according to the manufacturers instruction (DY493, R&D systems). BMP-9 ELISA and QPCR analysis were performed as described before (23, 24). Primers are described in supplementary table S1.

Cell culture and signaling assays

Primary human umbilical vein endothelial cells (HUVECs) were cultured as described before (25). P53/E-cadherin double knockout mouse breast cancer cells (KEP 1-11, (26)) were cultured as described before (27).

$3x10^5$ HUVEC cells were seeded in gelatin coated 6 well plates. Upon 90% confluency, cells were serum starved overnight in medium containing either 40 µg/mL TRC105 (Tracon Pharmaceuticals, San Diego, CA, USA) or 40 µg/mL human IgG (BioXCell, West Lebanon, NH, USA). 30 minutes before stimulation, 0.1% dimethyl sulfoxide (DMSO, as solvent control) or 1 µM SU5416 (WuXi app tec, Shanghai, China) was added, followed by a 10 minute stimulation with either 0.1 ng/mL recombinant human (rh)BMP-9 (R&D Systems, Abington, UK), 5 ng/mL TGF-β3 (28) or 50 ng/mL rhVEGF (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-β3. 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 orthovandate (BDH Laboratory, Poole Dorstet, UK)), protein content was determined and western blot analysis was performed as described before (24). Membranes were incubated overnight with primary antibodies against endoglin (R&D Systems), phosphorylated Smad1, phosphorylated Smad2, phosphorylated VEGFR2 Tyr1175 (all Cell Signaling technologies) or phosphorylated ERK1/2 (Sigma Aldrich). Blots were stripped and re-probed with either mouse anti-GAPDH (Millipore, Amsterdam, The Netherlands) or mouse anti-β-actin (Sigma Aldrich) antibodies as loading controls. Densitometry analysis was performed using ImageJ software.

MTS proliferation assay

HUVECs (2500 cells/well) were seeded in 96-well plates in triplicates. After 16 hours, medium was replaced with 100 µL medium supplemented with 40 µg/mL TRC105, 1 µM SU5416 or controls. Additionally, the assay was also performed in serum-free (SF) medium, containing bovine pituitary extract, and in the presence of 50 ng/mL rhVEGF or 0.1 ng/mL BMP-9. At indicated time points 20 µL MTS substrate (Promega, Madison, 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% FCS and 0.2% heparin. $2x10^4$ HUVECs were seeded in matrigel (BD Biosciences) coated 96-well plates in presence of 40 µg/mL TRC105, 1 µM SU5416 or controls. After 16 hour incubation, five images per well were acquired at 40x 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 (29). In short, HUVEC spheroids were embedded in collagen-I containing 0% or 20% FCS, and supplemented with 40 µg/mL TRC105, 1 µM SU5416 or controls. Images were acquired after overnight incubation and average sprout length was calculated using ImageJ software.

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 two day old Fli:eGFP zebrafish embryos. Two 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 hours 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).

Mouse breast cancer model

All animal experiments were approved by the Dutch animal ethics committee. Nine week old female Balb/c nu/nu mice (Charles River, L'Arbresle Cedex, France) were anaesthetized and $3x10^5$ luciferase expressing KEP1-11 cells were injected in the 4th mammary gland as described before (27). Mice were injected IP with 100 mg/kg luciferin, and imaged on the IVIS Lumina-II (Caliper Life sciences, Hopkinton, USA). Animals were treated twice weekly starting at week 2 after transplantation with 0.9% NaCl, 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, IP. Time between treatment with DMSO or SU5416 and human IgG or TRC105 was at least 24 hours. Tumor volume was measured twice weekly using a caliper and calculated (tumor volume = $(width^2 * length)/2$). After six weeks, mice were sacrificed and blood and tissue samples were collected.

For the tumor resection model, tumors were transplanted as described above and allowed to grow for four weeks. Mice were anaesthetized with KSA and the primary tumor was resected (30). Complete removal of the tumor was confirmed by bioluminescent imaging. Twice weekly adjuvant treatments with either 15 mg/kg human IgG or TRC105 were started one week after tumor resection (Fig. 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 µg/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 were sacrificed after 7 weeks (primary tumor) or 10 weeks (resection).

Statistical analysis

Differences between two groups were calculated using Student's t-test. Survival curves were generated using Kaplan-Meier analysis and the Log Rank test. P-values below 0.05 were considered significant.

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 neo-adjuvant 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. Pre-treatment biopsies (Fig. 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 (Fig. 1A, lower panel). To exclude that this observation was due to the difference of biopsies versus resection specimens, a group of patients that only received chemoradiation therapy (standard of care, Fig. 1B, upper panel) was compared with a group receiving both chemoradiation therapy and bevacizumab (Fig. 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 (HUVEC) were stimulated with VEGF or BMP-9, the main endoglin ligand. Treatment with BMP-9 induced rapid Smad1 phosphorylation, which could transiently be inhibited by TRC105 (Fig. 2A). In addition to inhibiting Smad1 phosphorylation, TRC105 increased phosphorylation of the anti-angiogenic Smad2 (Fig. 2B, left panel and supplementary Fig. S1A) similar to stimulation with 5 ng/mL TGF-β (Fig. 2B, right panel). Smad2 phosphorylation was dependent on ALK-5 kinase activity, since treatment with the kinase inhibitor SB431542 abrogated TRC105-induced Smad2 phosphorylation (Fig. 2B, right panel). These data indicate a shift towards an anti-angiogenic response by TRC105. VEGF induced rapid ERK phosphorylation in HUVECs, which could be inhibited by SU5416 (Fig. 2C). Interestingly, when HUVECs were treated with TRC105 and subsequently stimulated with VEGF, we observed increased ERK phosphorylation, compared to VEGF stimulation without TRC105 pre-treatment (Fig. 2D and supplementary Fig. S1B). This did not seem to be due to increased VEGFR2 Tyr1175 phosphorylation (supplementary Fig. 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 anti-angiogenic 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 (Fig. 3A). Under serum-free (SF) conditions (Fig. 3B), or SF-medium supplemented with either VEGF of BMP-9 (supplementary Fig. S2) we observed the same inhibitory effects on proliferation of HUVECs by SU5416 and combination treatment, albeit more pronounced. In contrast to BMP-9, VEGF enhanced

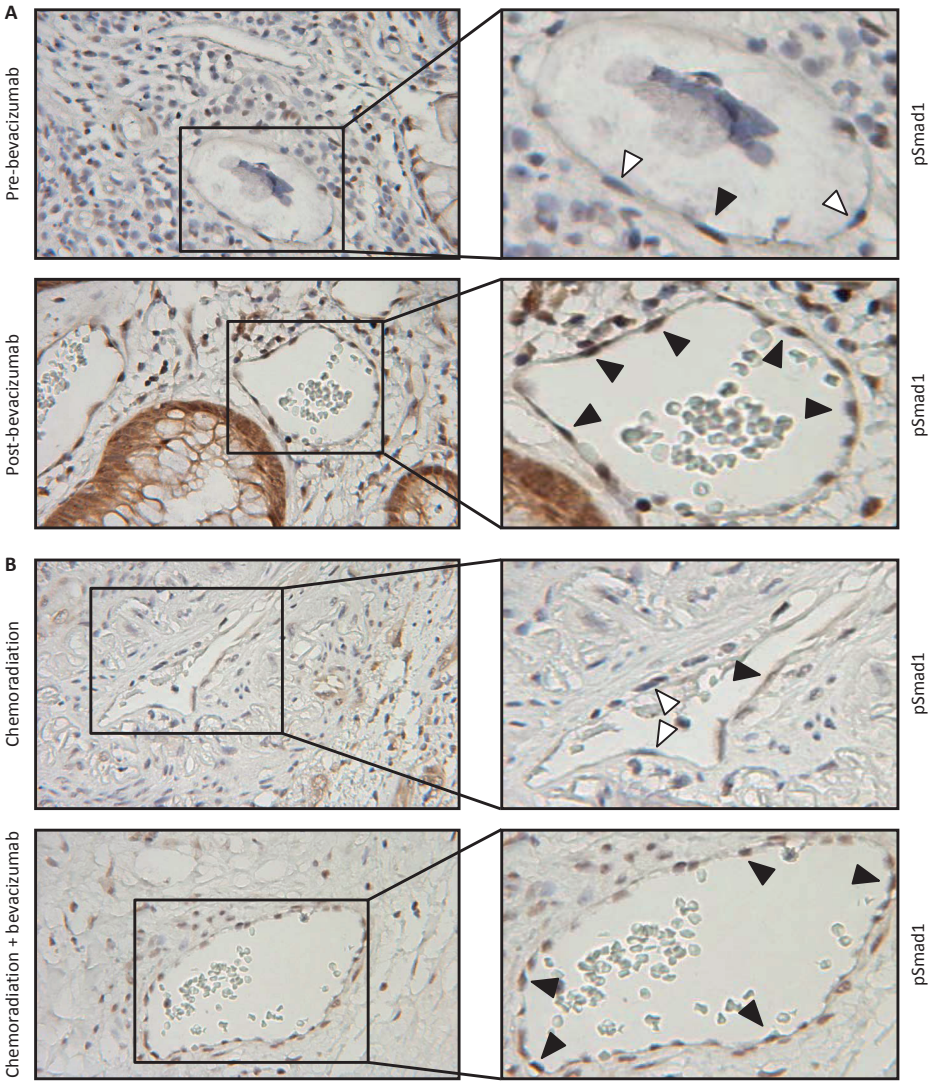


Figure 1 Anti-VEGF treatment results in increased Smad1 phosphorylation. A. Immunohistochemical staining for phosphorylated Smad1 (pSmad1) revealed increased endothelial nuclear pSmad1 in tissue samples from patients treated with bevacizumab (lower panel) compared with pre-treatment tumor biopsies (upper 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; pSmad1 negative nuclei. Black arrowheads; pSmad1 positive nuclei. Magnification x200, right panels x400.

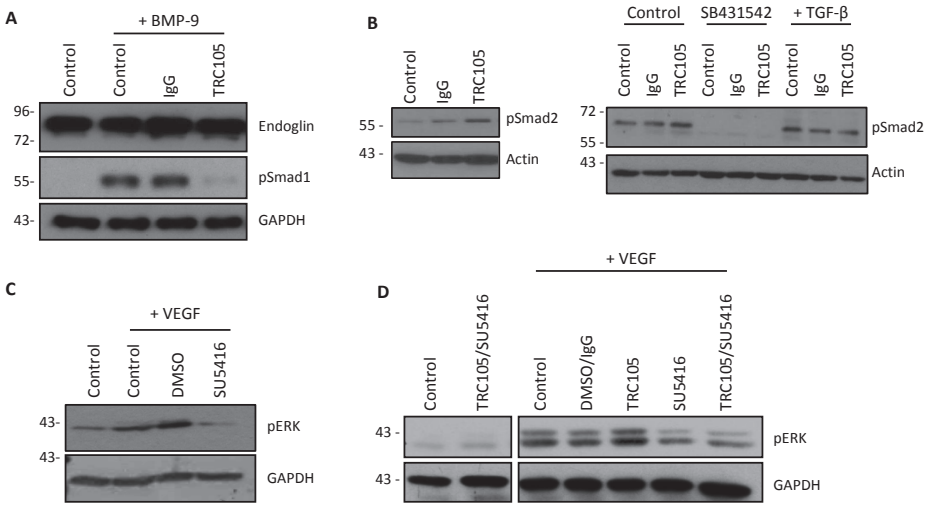


Figure 2 Redundancy between endoglin and VEGF signaling in HUVECs *in vitro.* A. BMP-9 stimulation (10 min) induced strong Smad1 phosphorylation (pSmad1) in HUVEC cells, which is inhibited by TRC105. B. Overnight treatment with TRC105 increased Smad2 phosphorylation (pSmad2, left panel), to a comparable level as induced by TGF-β (1h). This effect was completely blocked by the ALK-5 kinase inhibitor SB431542 (right panel). C. VEGF stimulation (10 min) increased ERK1/2 phosphorylation (pERK), which can be inhibited by SU5416. D. Pre-treatment with TRC105 increased VEGF-induced pERK, which can be reverted by SU5416 or TRC105/SU5416 combination therapy. All western blots are representative of three independent experiments.

HUVEC proliferation (Fig. 3C), underlining the importance of VEGF for endothelial cell proliferation *in vitro.*

Since, next to proliferation, migration of endothelial cells plays and additional role in angiogenesis, the cord forming ability of HUVECs in the presence of the inhibitors was assessed. First, we optimized the percentage of FCS in the medium for this assay, which was shown to be 5% (supplementary Fig. S3A). TRC105 did not significantly inhibit the number of closed polygons (Fig. 3D), nor the number of branches per branchpoint (supplementary Fig. S3B), while 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 which were formed were less dense and appeared to disintegrate faster than in the other conditions. Next, angiogenic potential in a more complex 3-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 Fig. S3C). TRC105 did not affect endothelial sprouting (Fig. 3E), confirming the results of the cord formation assay. A trend towards decreased average sprout length was observed for SU5416, while combination treatment resulted in statistically significant decreased HUVEC sprouting (Fig. 3E). These data indicate that endothelial sprouting is more efficiently inhibited by a TRC105/SU5416 combination.

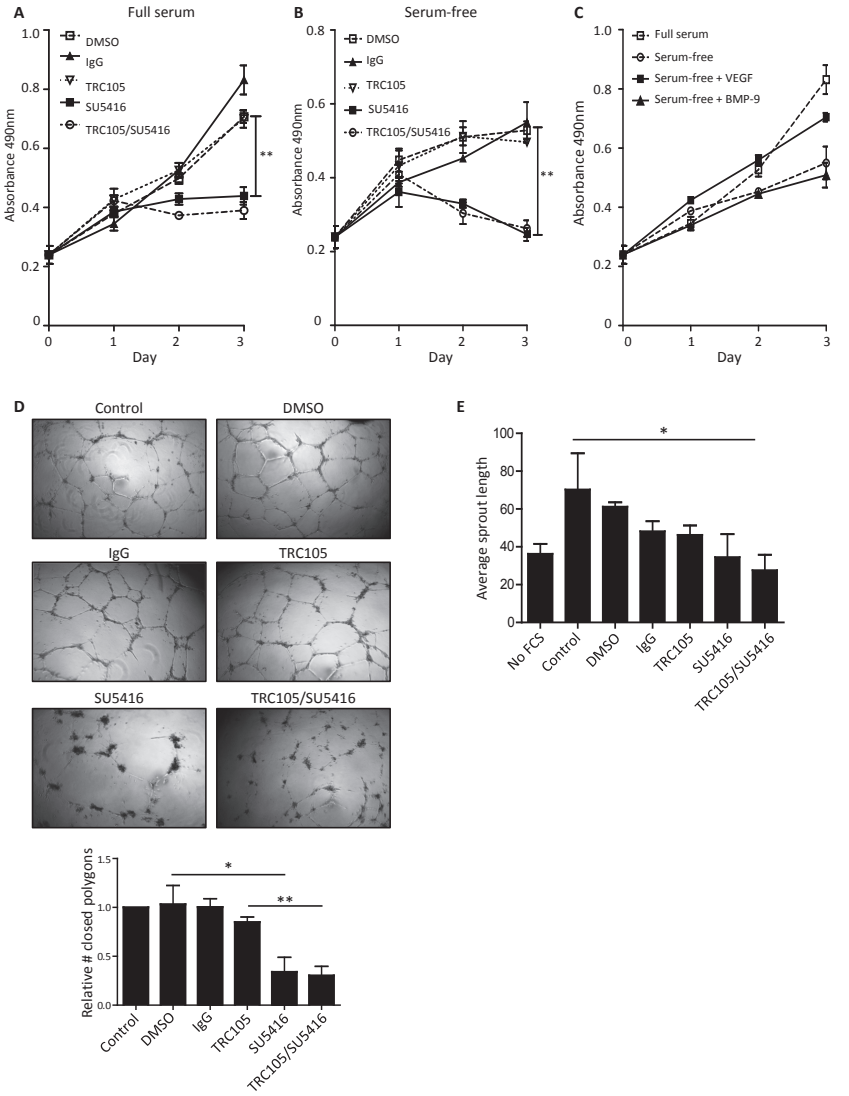


Figure 3 TRC105/SU5416 combination therapy enhances anti-angiogenic effects. TRC105 does not inhibit HUVEC proliferation in an MTS proliferation assay, while SU5416 and TRC105/SU5416 combination significantly decrease proliferation, in both full serum (A) and serum-free conditions (B). VEGF, either supplemented or BPE-derived, stimulates HUVEC proliferation, while 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 3-dimensional HUVEC sprouting assays sprouting of HUVECs was not affected by TRC105, while SU5416 and especially the TRC105/SU5416 combination significantly reduced sprouting. Data represent mean of three independent experiments, performed in triplicate. (*, P≤0.05, **, P≤0.01)

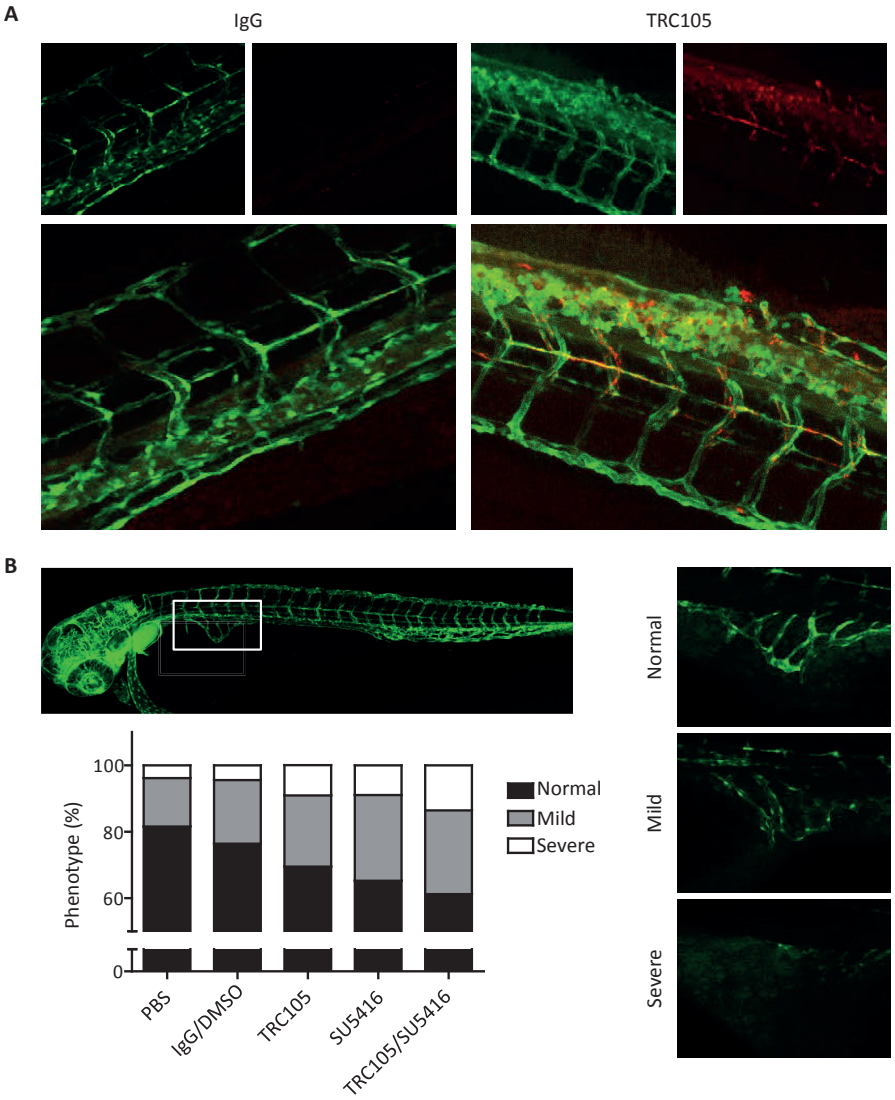


Figure 4 TRC105/SU5416 treatment inhibits angiogenesis in zebrafish. A. Two day old zebrafish embryos were injected with red fluorescently labeled human IgG or TRC105. TRC105 showed binding to GFP expressing zebrafish vasculature (right panel), whereas the IgG control did not show any fluorescent signal (left panel). B. Development of the subintestinal vessels (SIV) was assessed after two days of treatment with either TRC105, SU5416 or the combination. The SIV 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=38-114 fish/group/experiment).

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 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. Two days after injection, TRC105 (red) co-localized with the GFP-expressing zebrafish endothelium, whereas no red fluorescent signal was observed in the IgG control (Fig 4A), implying that TRC105 binds to the zebrafish endothelium. Next, we determined the anti-angiogenic effects of combination treatment. Zebrafish embryos were injected with TRC105, while SU5416 was applied via the fish water. Two days after treatment, the phenotype of the subintestinal vessels was scored as either normal, mildly, or severely affected (Fig. 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), Fig. 4B), while normal vessel development was decreased further after treatment with the TRC105/SU5416 combination (from 81.6% to 61.2%, Fig. 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. Luciferase expressing KEP1-11 mouse breast cancer cells (26) were transplanted into the mouse mammary gland. Tumor growth and metastasis were followed by *in vivo* bioluminescent imaging (BLI). The data showed that neither treatment with TRC105 nor SU5416, nor combination treatment affected tumor volume assessed by luciferase signal (Fig. 5A) or caliper measurement (Fig. 5B). Treatment with TRC105 or TRC105/SU5416 combination increased VEGF expression in tumor homogenates (supplementary Fig. S4A), whereas VEGF plasma levels were only increased upon TRC105 treatment (supplementary Fig. S4B). Using quantitative PCR, enhanced endoglin expression was observed after SU5416 and combination treatment (supplementary Fig. 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 (Fig. 5C/5D). 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 (SMA) positive cancer-associated fibroblasts (CAFs) revealed strongly reduced numbers of CAFs only in the TRC105 and combination treatment groups (Fig. 5E). Furthermore, a dramatic reduction in metastases was observed upon TRC105 monotherapy or in combination with SU5416 (Fig. 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.

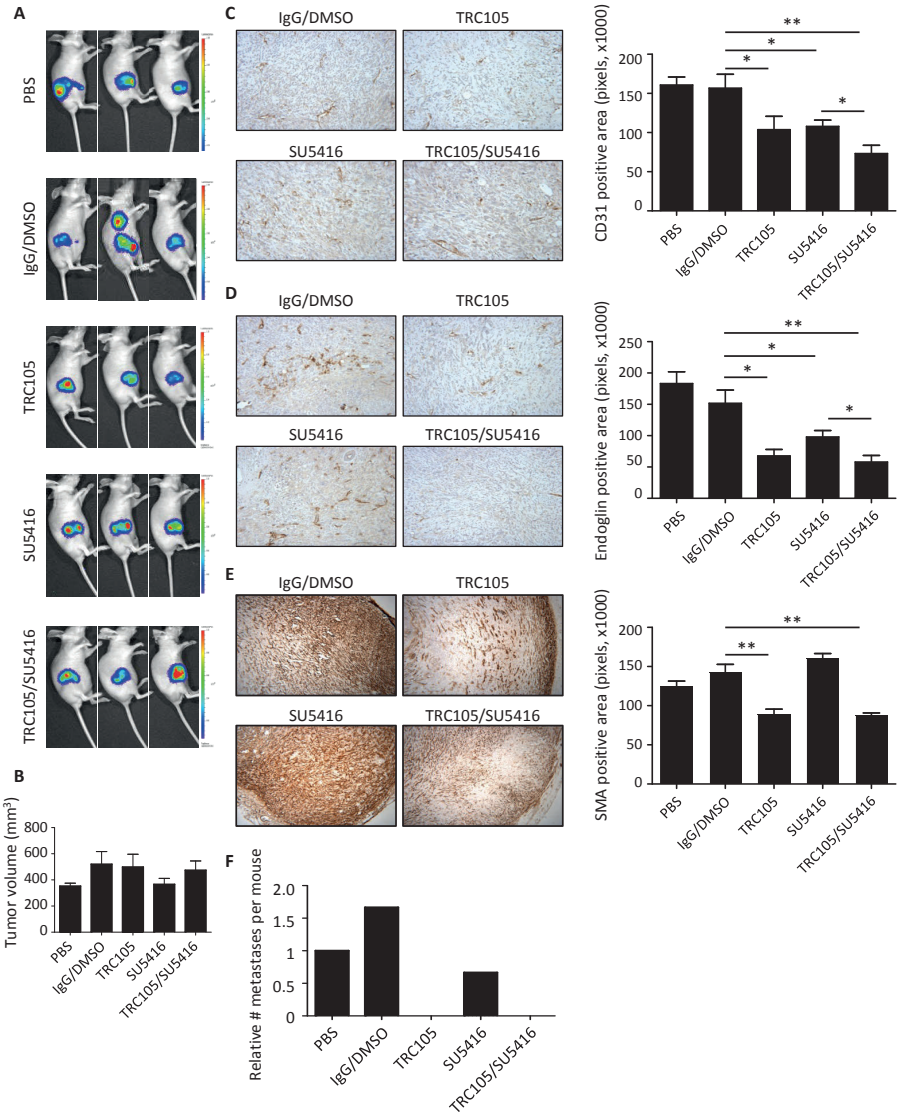


Figure 5 Combined targeting of endoglin and VEGF decreases tumor vascular density and metastatic spread of mouse breast cancer. KEP1-11 breast cancer cells were orthotopically transplanted and tumor growth was followed for six weeks using bioluminescent imaging (A) and caliper measurements (B). At the end of the experiment (6 weeks) tumor volume was not affected by the treatment, while 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. 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 (six weeks after start of experiment, n= 4-6 mice/group). (*, P≤0.05, **, P≤0.01)

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. Four weeks after transplantation, the primary tumor was removed and mice received adjuvant treatment with TRC105 or human IgG (Fig. 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 BLI. Six weeks after resection, BLI showed a high number of metastases in the IgG control group, which was strongly reduced in the TRC105 treated group (Fig. 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%, Fig. 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). Additionally, morphology of the primary tumor and metastases was similar in both treatment groups (supplementary Fig. 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,

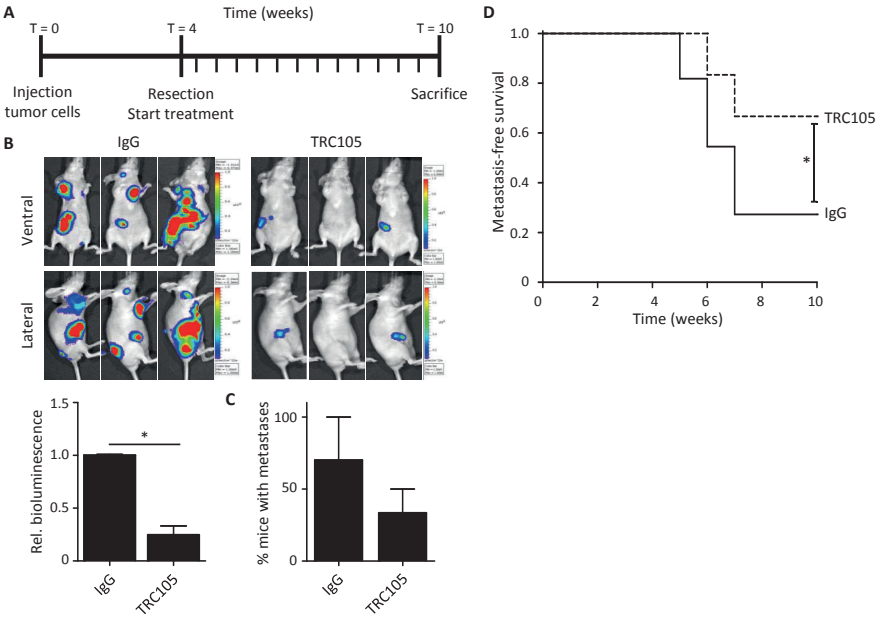


Figure 6 TRC105 treatment inhibits breast cancer metastasis an in adjuvant setting. A. Experimental set-up. Four weeks after tumor transplantation, primary tumors were resected and mice were treated with human IgG or TRC105 for six additional weeks. B. Metastasis formation was followed over time using 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%, compared to 75% in the control mice (C). This results in significantly improved metastasis-free survival of mice treated with TRC105 compared to IgG treatment group (D). Graphs represent mean of 11-12 mice/group from 2 independent experiments. (*, P≤0.05)

bone and peritoneal wall. Only four out of 12 mice treated with TRC105 showed distant metastases, resulting in significantly improved metastasis-free survival of this group (Fig. 6D). These data indicate that adjuvant treatment with TRC105 prevents the development of distant breast cancer metastases.

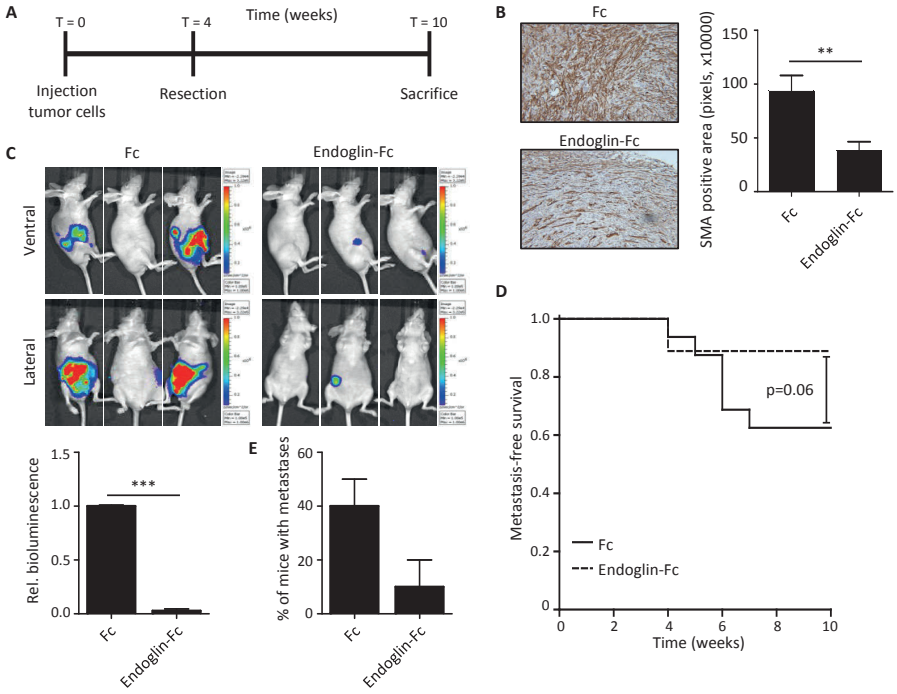


Figure 7 Endoglin-Fc expression decreases metastatic spread of breast cancer cells. A. Experimental set-up. Four weeks after tumor transplantation of cell lines stably expressing Fc or Endoglin-Fc, tumors were resected and metastatic spread was followed for six additional weeks. B. SMA+ stroma content 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 more than 50% compared with Fc. Graphs represent mean of 8 mice/group from 2 independent experiments. (**, P≤0.01, ***, P≤0.001)

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 Fig. 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 Figs. S6B and S6C), while endoglin-Fc strongly decreased primary tumor vascularization (supplementary Fig. S6D). Interestingly, like treatment with TRC105,

mice with endoglin-Fc expressing tumors exhibited a reduced number of metastases (supplementary Fig. 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 four weeks. Primary tumors were removed and mice were monitored for an additional 6 weeks (Fig. 7A). Upon resection the number of SMA+ CAFs in the primary tumors was significantly lower in the endoglin-Fc group compared with the Fc control group (Fig. 7B). At the end of the experiment bioluminescent signals in the endoglin-Fc group were significantly reduced, compared with the Fc group (Fig. 7C). Expression of endoglin-Fc resulted in decreased metastatic spread and therefore improved metastasis-free survival (Fig. 7D). Additionally, the percentage of mice showing distant metastases decreased by more than 50% in the mice bearing endoglin-Fc expressing tumors (Fig. 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 HUVEC cells. Contrary to our observations, Liu et al. described a 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 (31). 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 (32). 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 efficacy (33) and might lead to enhanced effects.
Although xenograft studies showed very promising results for anti-angiogenic 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 (34). However,

at the end of the study, the same pro-angiogenic 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, which unfortunately did not result in decreased tumor volumes. Treatment with bevacizumab, can result in vessel normalization (35, 36). Leaky and irregular tumor vessels partly disappear, but the remaining vessels gain "normal" morphology and pericyte coverage (37). This results in increased blood and nutrient supply to the tumor, sustaining tumor growth, but also enhanced effects of chemotherapy on breast cancer as we could recently show for anti-angiogenic therapy targeting ALK1 (27).
Recently, the first clinical trial assessing TRC105 and bevacizumab combination in patients has been published (21). Of the 38 patients enrolled in this trail, 18 patients showed at least a partial response to treatment. 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 anti-angiogenesis adverse effects upon TRC105 monotherapy (38). 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. reported that endoglin heterozygous mice show increased liver metastasis in Rip-Tag mice (39). Endoglin heterozygote mice develop a hereditary hemorrhagic telangiectasia (HHT) phenotype including vascular malformations (40) and increased vascular permeability (41) possibly resulting in enhanced metastatic spread. When we treated endothelial monolayers with TRC105, trans-endothelial 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 (42). 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. Since 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* (43), hardly express endoglin or VEGFR2 (supplementary Fig. S7), therefore direct targeting of the tumor cells in our model

is unlikely. However, besides endothelial cells, endoglin is also expressed on 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 (38). Additionally, macrophages can express endoglin (44), in which it can direct macrophages in either a tumor-suppressive M1 or tumor-promoting M2 phenotype (45).

Our study revealed that treatment with TRC105 or expression of endoglin-Fc resulted in decreased SMA-positive CAF content of the tumors (Figure 5E/7B). The percentage of tumor stroma is known to be a prognostic marker in breast cancer (46, 47). More specifically, CAFs have been shown to play an important role in breast cancer progression and metastasis (reviewed in (48)). We and others (42) 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, which 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 anti-angiogenic responses *in vitro* and *in vivo,* compared to 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.

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Conflict of interest

C. Theuer is full time employee of Tracon Pharmaceuticals. All other authors declare no conflicts of interest.

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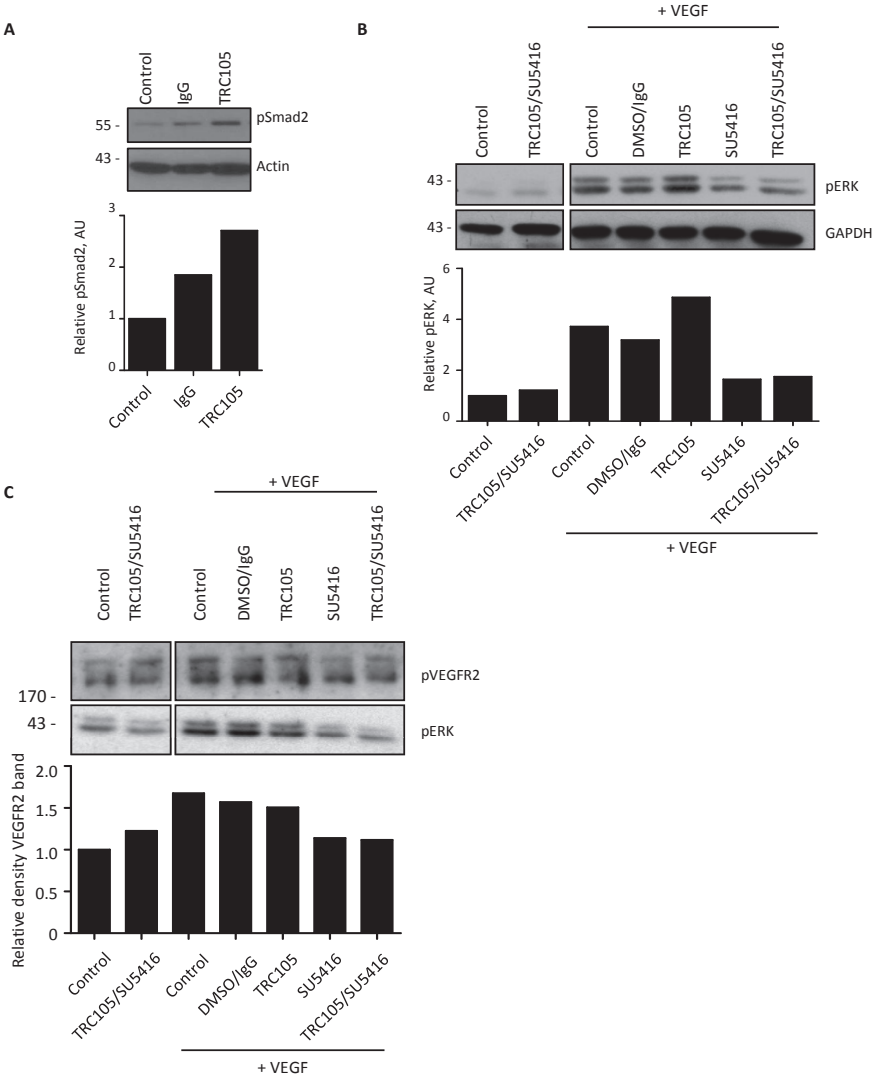
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Supplementary data

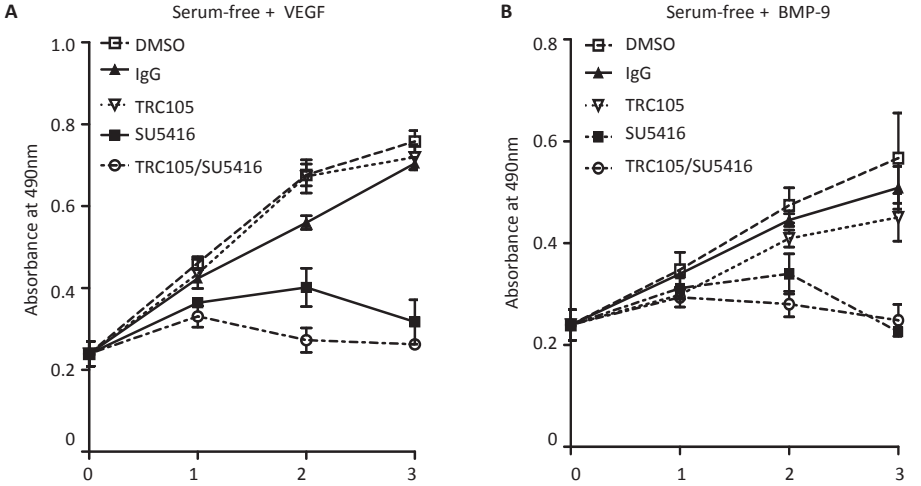


Supplementary figure S1 Densitometric quantification of pSmad2, pERK and pVEGFR2 Tyr1175. A. Overnight treatment with TRC105 increased basal Smad2 phosphorylation in HUVECs. B. Pre-treatment with TRC105 increased VEGF-induced ERK phosphorylation after 10 minutes of stimulation in HUVEC. C. Pre-treatment with TRC105 did not result in increased VEGFR2 phosphorylation on tyrosine1175. Quantifications were corrected for loading control.

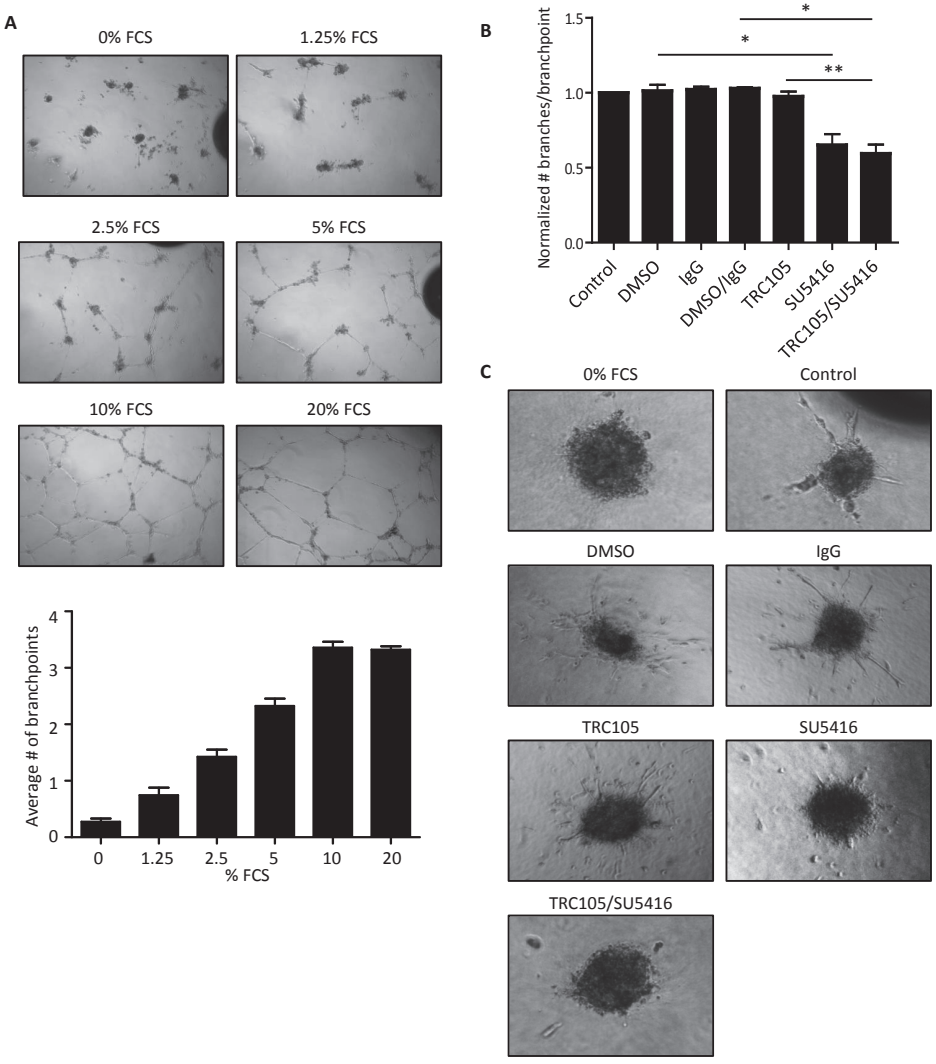
Supplementary table S1 QPCR primer sequences

Gene	Forward primer	Reverse primer
Endoglin	tgaatggcaaccacgagc	gcaggatgagaatggcgtc
VEGFR2	tgcgaagccaccgtcaa	ctcgggcgggcgtatttg
GAPDH	atcactgccacccagaagac	atgaggtccaccaccctgtt

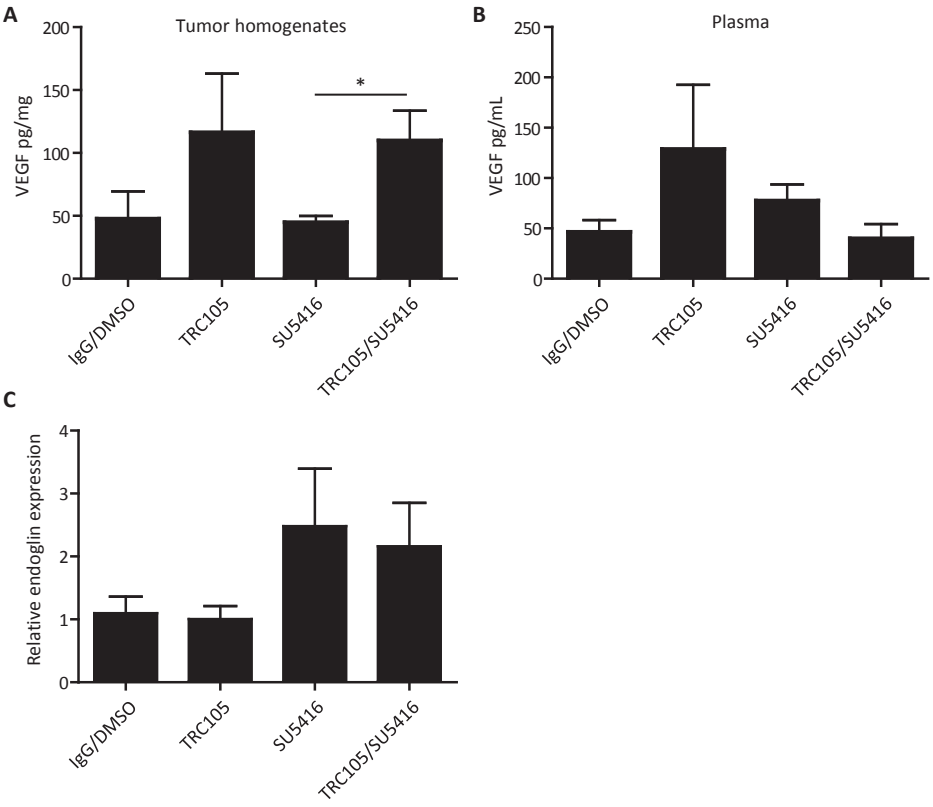
QPCR primers as used for detecting mouse endoglin, mouse VEGFR2 and mouse GAPDH.



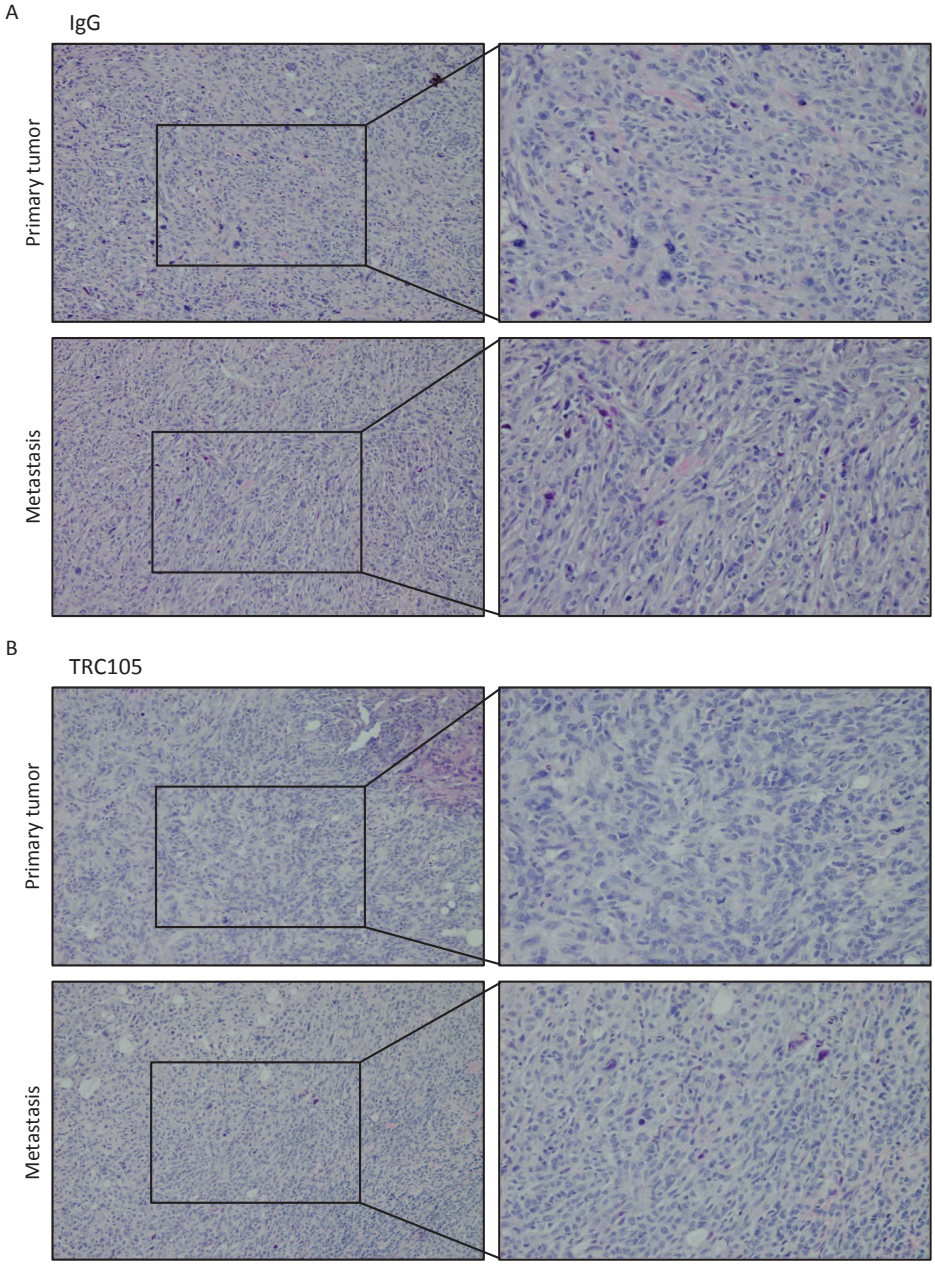
Supplementary figure S2 HUVEC proliferation is dependent on VEGF, but not on the presence of BMP-9. In order to determine the effects of VEGF and BMP-9 on HUVEC proliferation, MTS proliferation assays were performed using serum-free medium (containing BPE). A. When serum-free medium was supplemented with VEGF, HUVEC proliferation was enhanced when compared to serum-free conditions, showing VEGF-dependency. Additionally, as in full serum conditions, targeting VEGF signaling with SU5416 or the combination treatment inhibited proliferation. B. BMP-9 did not affect HUVEC proliferation, although SU5416 and combination treatment still inhibit proliferation in this condition. Graphs are representatives of two independent experiments performed in triplicate.



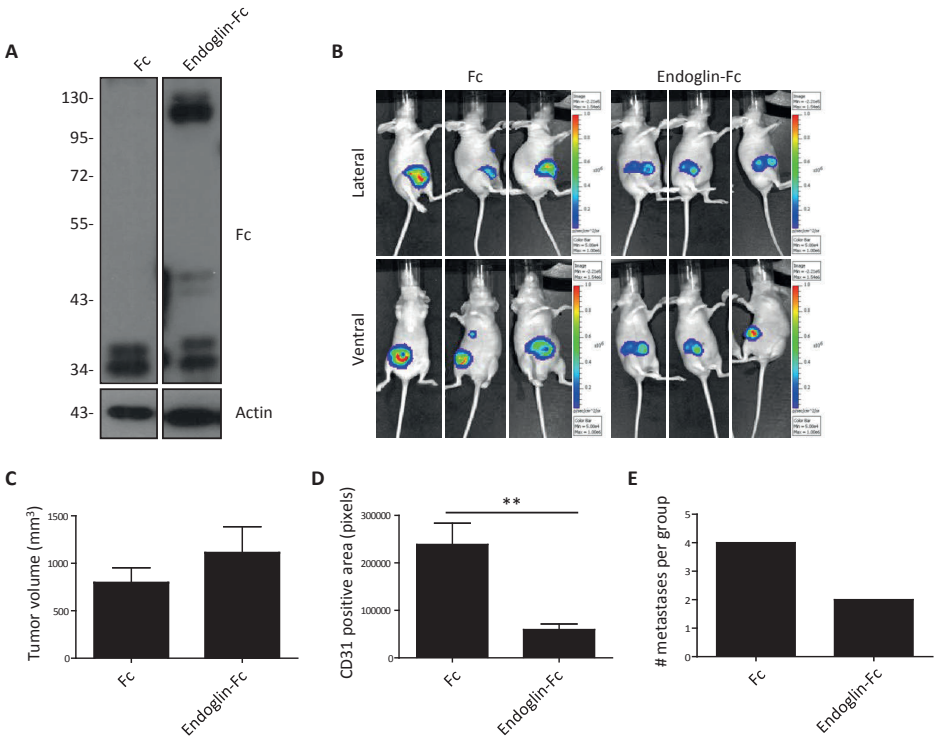
Supplementary figure S3 HUVEC *in vitro* angiogenesis assays. A. To determine the minimal percentage of FCS in the medium required to observe cord formation, a concentration range of FCS was tested. Based on these experiments, 5% FCS was the lowest concentration at which HUVEC cord formation occurred. B. 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 branches per branchpoint. Data are normalized to control and represent mean of at least 2 independent experiments, performed in triplicate. C. In 3-dimensional HUVEC sprouting assays, sprouting of HUVECs was not affected by TRC105, while SU5416 and especially the TRC105/SU5416 combination significantly reduced sprouting. Data represent mean of three independent experiments, performed in triplicate. (* P≤0,05; ** P ≤0,001)



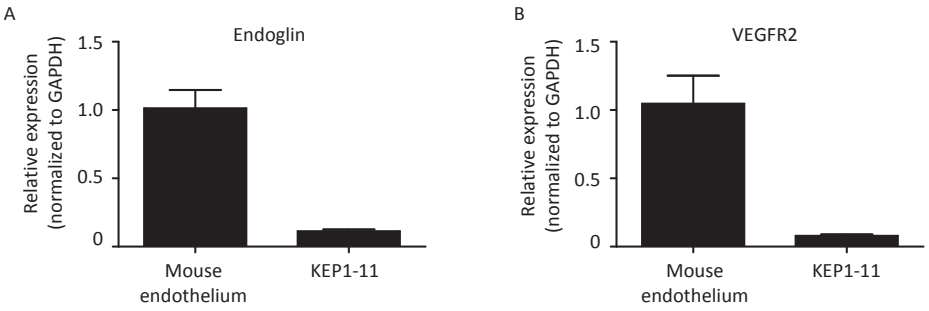
Supplementary figure S4 VEGF and endoglin levels in mouse samples. A. ELISA analysis was performed on tumour homogenates prepared in RIPA lysis buffer. Treatment with TRC105, alone or in combination with SU5416, resulted in increased tumour VEGF levels. B. ELISA analysis on mouse plasma samples. Plasma VEGF levels were increased upon TRC105 treatment, which was reverted after TRC105/SU5416 combination treatment. C. Endoglin mRNA levels were determined using quantitative PCR. Increased endoglin mRNA expression in the groups treated with SU5416, either as monotherapy or in combination with TRC105. (n=4-6 mice/group; *, P≤0.05)



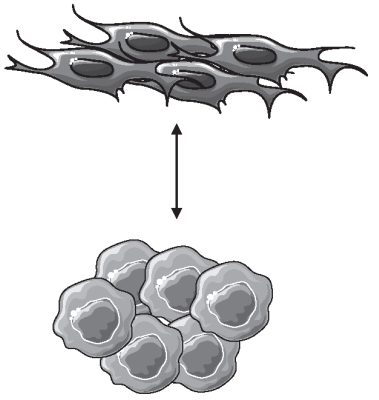
Supplementary figure S5 Histology of primary tumor and metastases. Primary tumors were resected four weeks after transplantation of KEP1-11 cells and metastases were obtained at the end of the experiment. Morphology of primary tumors and metastases were assessed for both the IgG control group (A) and TRC105 treated mice (B).



Supplementary figure S6 Endoglin-Fc expression inhibits breast cancer metastasis. KEP1-11 cells were transduced with Fc and endoglin-Fc lentiviral vectors and stable cell lines were created. Expression of the constructs was confirmed using anti-Fc western blot (A). Tumor growth was monitored using bioluminescence (B) and caliper measurement (C). No difference in primary tumor growth was observed, while CD31 immunohistochemical staining revealed a significant decrease in tumor vascular density upon endoglin-Fc expression (D). Mice with endoglin-Fc expressing tumors show reduced number of metastases per group when compared with Fc. (E, n=8 mice/group; **, P≤0.001)



Supplementary figure S7 Endoglin and VEGFR2 mRNA expression in KEP1-11 cells. mRNA expression of endoglin (A) and VEGFR2 (B) by KEP1-11 cells was assessed using quantitative PCR and compared to mouse endothelial cells. Data were normalized to GAPDH expression.



Chapter 6

Interaction with colon cancer cells hyperactivates TGF-β signalling in cancer-associated fibroblasts

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Abstract

The interaction between epithelial cancer cells and cancer-associated fibroblasts (CAFs) plays a major role in cancer progression and eventually in metastasis. In colorectal cancer, CAFs are present in high abundance, but their origin and functional interaction with epithelial tumour cells has not been elucidated. In this study we observed strong activation of the TGF-β/Smad signalling pathway in colorectal cancer CAFs accompanied by decreased signalling in epithelial tumour cells. We evaluated the TGF-β1 response and the expression of target genes including *matrix metalloproteinases (MMPs)* and *plasminogen activator inhibitor (PAI)-1* of various epithelial colorectal cancer cell lines and primary CAFs *in vitro*. TGF-β1 stimulation caused high up-regulation of MMPs, PAI-1 and TGF-β1 itself. Next we showed that incubation of CAFs with conditioned medium from epithelial cancer cells led to hyperactivation of the TGF-β signalling pathway, enhanced expression of target genes like *PAI-1*, and the expression of α-*smooth muscle actin (*α-*SMA)*. We propose that the interaction of tumour cells with resident fibroblasts results in hyperactivated TGF-β1 signalling and subsequent transdifferentiation of the fibroblasts into α-SMA-positive CAFs. In turn this leads to cumulative production of TGF-β and proteinases within the tumour microenvironment, creating a cancer-promoting feedback loop.

Introduction

The interaction between carcinoma cells and fibroblasts within the tumour microenvironment contributes to cancer initiation, progression and eventually metastasis in many cancer types (1;2). Cancer-associated fibroblasts (CAFs) are a heterogeneous group of fibroblasts expressing vimentin, fibroblast-activating protein (FAP) and α-smooth muscle actin (α-SMA) (3-6). Compared to normal mucosa the number of myofibroblasts is strongly increased in colorectal cancer (5;7), where they influence the immune response and show increased synthesis of chemokines, cytokines, proteolytic enzymes and several extracellular matrix (ECM) components (8). The origin of CAFs has not fully been clarified yet (6), but based on primarily *in vitro* studies, the most commonly accepted hypothesis is that the majority of CAFs are generated by Transforming Growth Factor-β (TGF-β) induced trans-differentiation of resident fibroblasts (1;9). In addition to its role in CAF formation, TGF-β is involved in tumour progression/metastasis by inducing tumour angiogenesis, increasing production of ECM, proteolytic enzymes and immune suppression (10-12).

TGF-β is synthesized as a latent precursor consisting of a TGF-β homodimer non-covalently linked to the latency associated protein (LAP), forming the small latent complex (SLC). The latent TGF-β binding protein (LTBP) connects the complex to the ECM as the large latent complex (LLC) (13) and is important for TGF-β function (14). TGF-β activation involves removal of both LTBP and LAP and occurs through proteolytic processing (e.g. plasmin (15) and matrix metalloproteinases (MMPs) (16)) or non-proteolytically via conformational changes (e.g. integrins (17)). The activating mechanism seems to be strongly dependent on the cell/tissue type or the experimental conditions. Active TGF-β binds to the widely expressed TGF-β type-II receptor (TβRII), which subsequently recruits and transphosphorylates the type-I TGF-β receptor activin receptor-like kinase (ALK)-5. Subsequently, intracellular signalling is initiated via phosphorylation of Smad2/Smad3 and recruitment of Smad4. Smad complexes translocate to the nucleus, where they regulate transcriptional responses (18).

In this study we investigated the interaction between epithelial tumour cells and CAFs with respect to TGF-β signalling and consequent regulation of proteinase and α-SMA expression. First, α-SMA expression and TGF-β1 activity were immunohistochemically studied in human colorectal cancer specimens. Next we assessed TGF-β1 response of both epithelial tumour cells and CAFs, the regulation of TGF-β target genes and proteinases implicated in invasion, and TGF-β activation. Our data show that epithelial tumour cell derived TGF-β hyperactivates TGF-β signalling in fibroblasts, leading to the generation of CAFs, accompanied by increased TGF-β1 and MMP production, creating a cancer-progressing feedback loop.

Materials and methods

Patient samples

Tissue specimens from patients undergoing surgical resection for CRC at the Department of Surgery, Leiden University Medical Centre, were collected. Tissues were formalin-fixed, dehydrated, and embedded in paraffin. The patient population contained stage I-III CRC patients and is part of the cohort described before(19). Fresh tissues from the patients were used for isolation of primary CAFs. The study was performed according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Centre.

Immunohistochemistry

Immunohistochemical staining was performed as described before (32). In short, sequential sections were deparaffinised, rehydrated and endogenous peroxidase activity was quenched. Antigen retrieval was performed by boiling in 0.01 M sodium citrate, pH 6.0 for 10 minutes followed by overnight incubation at room temperature (RT) with primary antibodies described in table 1. Sections were incubated with biotinylated secondary antibodies, streptavidin-biotin complex (all Dako, Denmark) and staining was visualized using diaminobenzidine and H_2O_2. Representative photomicrographs were taken with a Nikon Eclipse E800 microscope equipped with a Nikon DXM1200 digital camera.

Table 1 Antibodies used for immunohistochemistry.

Antibody	Clone	Manufacturer	Final concentration	Antigen retrieval
Pan-Cytokeratin	C11	Santa Cruz Biotechnologies, USA	200 ng/ml paraffin 40 ng/ml cytospins	Citrate[1]
Vimentin	V9	Santa Cruz, Biotechnologies, USA	500 ng/ml paraffin 200 ng/ml cytospins	Citrate[1]
SMA	ASM-1	Progen, Heidelberg, Germany	130 ng/ml paraffin 33 ng/ml cytospins	Citrate[1]
pSmad2	Rabbit monoclonal	Cell signalling technologies, USA	1:100 paraffin	Citrate[1]
TGF-β	TB21	Anogen, USA	1 μg/ml	Citrate[1]

[1] Boiling in 0.01 M Sodium Citrate buffer pH 6.0, 10 minutes

Cell culture, isolation of CAFs and preparation of conditioned media (CM)

HT29, HCT116, LS180, Lovo, SW480, SW948, and CaCo-2 cells were cultured in DMEM/F12 + GlutaMAX-1 (Invitrogen, the Netherlands), 10 mM HEPES, 50 μg/ml gentamycin, 100 U/ml penicillin and 100 μg/ml streptomycin (all Invitrogen), supplemented with 10% heat inactivated Fetal Calf Serum (FCS, Perbio Science, Belgium) or 20% FCS for CaCo-2 cells. Human CAFs were isolated from the non-necrotic part of the tumours. The tissue was washed with PBS, cut into 5 mm pieces and incubated in tissue culture flasks. After 7-10 days fibroblast-like cell outgrowth was observed. The fibroblast origin of these cells was confirmed by positive staining for vimentin and negative staining for pan-cytokeratin. CAFs

were maintained in complete DMEM/F12 containing 10% FCS and used at passage 5-11. Conditioned media (CM) from CRC cell lines and CAFs were prepared by seeding the cells and growing them to sub-confluence. Medium was changed to serum free (SF)-DMEM/F12, containing HEPES and antibiotics as described above, and incubated for 4 days. CM used for stimulation was diluted 2-fold with fresh SF-DMEM/F12. To obtain myofibroblast CM, fibroblasts were stimulated with 5 ng/ml recombinant human TGF-β1 (Peprotech, United Kingdom) for 24h. After three washes with SF-DMEM/F12, cells were incubated for 4 days with SF-DMEM/F12. Medium was analysed for MMP-2 and MMP-9 levels by zymography as described before (42) and for TGF-β1 levels by ELISA as described below.

MTS proliferation assay

HT29 and HCT116 cells were seeded in 96-well plates (2,000 or 10,000 cells/well) and allowed to attach overnight. Cells were stimulated with 5 ng/ml TGF-β1 for 6h or 72h. Subsequently the medium was changed to 100 µl complete DMEM/F12 + 20 µl MTS substrate (Promega, USA). The metabolic activity of the cells was analysed by absorbance change at 490 nm after 2h.

TGF-β response assay

TGF-β response in tumour cells was determined using $(CAGA)_{12}$-MLP-Luciferase promoter reporter construct (43). This construct contains 12 palindromic repeats of the Smad3/4 binding element derived from the *PAI-1* promoter and was shown to be highly specific and sensitive to TGF-β. Tumour cells were seeded in 24-well plates and allowed to attach overnight. Subconfluent cells were transfected using Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol. A β-galactosidase plasmid was co-transfected to correct for transfection efficiency. After 6h medium was changed to complete DMEM/F12 and the cells were incubated for 24h and serum starved overnight. Cells were stimulated with 0-5 ng/ml TGF-β1 or indicated conditions. After stimulation the cells were washed, lysed and luciferase activity was determined according to the manufacturer's protocol (Promega). β-galactosidase activity in the lysates was determined using β-gal substrate (0.2 M H_2PO_4, 2 mM $MgCl_2$, 4 mM ortho-nitrophenyl-phosphate, 0.25% β-mercaptoethanol) and measuring absorbance change at 405 nm. The luciferase count was corrected for β-galactosidase activity. The relative increase in luciferase activity was calculated versus controls.

CAFs were infected using an adenoviral Ad-$(CAGA)_9$-MLP-Luc promoter reporter construct (44). Cells (22,000/well) were infected with 1E+6 pfu virus. After infection medium was changed to complete DMEM/F12 for 24 hours and the CAFs were serum starved overnight, stimulated, lysed and the luciferase activity was determined as described above.

Western blot analysis

Western blot analysis was performed as described before (45). In short, equal protein amounts (DC protein assay, Biorad laboratories, USA) were separated on 10% SDS-PAGE

under reducing conditions. Proteins were transferred to nitrocellulose membranes (Whattman, Germany) and unspecific binding was blocked with 5% milk powder in TBS containing 0.05% Tween-20 (TBST, Merck, Germany). Blots were incubated overnight with mouse anti-Smad4 antibodies (Santa Cruz biotechnologies, USA), rabbit anti-TβRII (Santa Cruz), rabbit anti-pSmad2 (46), rabbit anti-pSmad3 (kindly provided by Dr. E. Leof (Mayo Clinic, Rochester, USA)), rabbit anti-PAI-1 (Santa Cruz), mouse anti-β-actin (Sigma-Aldrich, USA). Detection was performed by HRP-conjugated secondary antibodies (all GE Healthcare, USA) and chemoluminescence according to the manufacturer's protocol (Pierce, USA). Blots were stripped and reprobed with anti-β-actin antibodies as a loading control. To detect latent TGF-β complexes in CM, 10-50 µl CM was run under non-reducing conditions. Blots were probed with anti-LAP (R&D systems) or anti-TGF-β1 (Promega) antibodies and analysed as described above.

RNA isolation and real-time PCR

Expression of TGF-β target genes and MMPs were analysed in HT29, HCT116, SW948 and CaCo-2 cells. Cells were grown to subconfluence and stimulated with 5 ng/ml TGF-β1 or 2 fold diluted CM for indicated time points, harvested and RNA was isolated using RNeasy Mini Kit according to the manufacturers' instructions (Qiagen, USA). RNA concentration and purity was determined spectrometrically. cDNA synthesis was performed on 1 µg RNA using random primers. cDNA samples were subjected to 40 cycles Real-Time PCR analysis using primers as previously described (47;48), except for *MMP-7*, *MMP-13* and *MMP-28* primer sets which were purchased from Applied Biosystems, the Netherlands. *PAI-1* qPCR analysis was performed as described before (49). All values were normalised for cDNA content by *GAPDH* or *actin* expression. TGF-β does not influence GAPDH expression (50).

TGF-β ELISAs

Active TGF-β1 and total TGF-β1, TGF-β2 and TGF-β3 levels in CM samples were determined by commercially available duo-sets (R&D systems) as described before (32;34).

Spheroid invasion assays

HT29 or CAF spheroids were grown as described before (51). Spheroids were collected and embedded in collagen gels, consisting of DMEM/F12 with 0-10 ng/ml (0-400 pM) TGF-β1 and 1 mg/ml collagen type 1 (Vitrogen, Nutacon, USA). HT29 spheroids were analyzed at 4-14 days for invasiveness and the formation of distant metastasis-like cell clusters. CAF spheroids were embedded and treated with 5 ng/ml TGF-β1 or 10 µM GM6001 and analysed after 1 and 2 days for invasion. Representative photomicrographs were taken using a Zeiss Axiovert microscope equipped with 10x objective.

TGF-β1 activation experiments

To analyze TGF-β1 activation by tumour cells and CAFs these cells were transfected or transduced as described above. Cells were stimulated with 10 ng/ml (121 pM) rh-small latent TGF-β1 complex (R&D systems), tumour cell CM or CAF-CM.

To examine the contribution of various proteins described to be involved in TGF-β1 activation, CAFs were stimulated with HCT116 CM in the presence of: 10 μM ALK-5 inhibitor SB431542 (Tocris, USA), a TGF-β neutralising antibody (R&D systems, 10 μg/ml), 1 μM GM6001 (broad spectrum MMP inhibitor), 1 μM specific MMP-2/9 or specific MMP-13 inhibitor, 10 μM specific MMP-3 inhibitor (all Calbiochem, USA), 1 μM Marimastat (broad spectrum MMP inhibitor, kindly provided by British Biotech Pharmaceuticals, UK), 10 μg/ml aprotinin (serine protease inhibitor including plasmin), 20 μM E64 (cystein protease inhibitor), 100 nM α-2 macroglobulin (all Sigma-Aldrich) 100 μM RGD (H-Arg-Gly-Asp-OH) peptide (kindly provided by Dr. E. de Heer, LUMC, Dept. Pathology) or 2.5 mM NAC (n-acetyl-cysteine, a reactive oxygen species inhibitor, Sigma-Aldrich). Combinations of inhibitors (Inhibitor cocktail-1: MMP-2/-9,-3,-13 inhibitors, aprotinin and E64; inhibitor cocktail-2: aprotinin, GM6001 and E64) were also tested. After 6h incubation luciferase activity was determined as described above. For CAF experiments parallel incubations were performed, medium was harvested for the TGF-β1 ELISA and cells were harvested by trypsinisation. Cytospins were prepared and cells were analysed for α-SMA content by immunocytochemistry.

Results

TGF-β signalling in colorectal cancer (CRC) tissues

To analyse TGF-β signalling in CRC we stained 88 CRC tissues and normal colonic mucosa for TGF-β signalling components. In normal mucosa a single layer of vimentin positive pericryptal cells co-stained for α-SMA indicating a myofibroblast phenotype (Figure 1A, upper panel). Staining for TGF-β1 was low in normal mucosa, while epithelial cells did show nuclear phosphorylated-Smad2 (pSmad2) staining, indicating active TGF-β signalling. Nuclear pSmad2 staining showed an increasing gradient from bottom to top of the crypt (Figure 1A), consistent with the anti-proliferative and pro-apoptotic features of TGF-β in normal tissue. pSmad2 was barely detectable in normal fibroblasts or myofibroblasts.

In contrast, TGF-β staining in CRC tissues was strongly enhanced in malignant epithelial cells and tumour stroma. The majority of fibroblasts (up to 90%) was of the α-SMA-positive myofibroblast phenotype and in 89% of the patients we observed nuclear pSmad2 accumulation (Figure 1A, lower panel). Moreover, absent epithelial nuclear pSmad2 was observed in 58% of the CRCs, indicating a shift from primarily epithelial TGF-β signalling in normal tissues to mesenchymal signalling in tumours (Figure 1B, upper panel). In contrast the lower panel in Figure 1B shows a representative picture of 42% of the tumours in which

the malignant epithelial cells still displayed nuclear pSmad2 localization, in addition to the surrounding CAFs.
Next we analysed the relationship between epithelial presence of pSmad2 and survival of CRC patients. These data revealed that patients with epithelial pSmad2 have a significant worse disease-free survival compared to patients with absent epithelial pSmad2 (Log rank 3.78, p=0.05, Figure 1C).

Characterisation of TGF-β response in CAFs

To examine the TGF-β response in CAFs, we isolated CAFs from five CRC tissues. The number of α-SMA-positive CAFs was evaluated by immunocytochemistry and varied per tumour between 32% and 92% (Supplementary figure 1A). All CAFs showed a dose-dependent TGF-β transcriptional response (up to 100-fold) as shown in Supplementary figure 1B and a representative graph in Figure 2A. TGF-β induced Smad2 phosphorylation was also seen in all CAFs tested (Supplementary figure 1C). Real-time PCR analysis revealed high basal expression of *collagen type 1* and *plasminogen activator inhibitor (PAI)-1*. Expression of *matrix metalloproteinases* (*MMPs*) in CAFs was generally high compared to CRC cells (Table 2). TGF-β1 treatment increased the expression of *collagen-1*, *PAI-1*, *urokinase type plasminogen activator* (*uPA*), various *MMPs* and *tissue inhibitors of matrix metalloproteinases* (*TIMPs*) in CAFs (Figure 2B). Interestingly, the expression of *TGF-β1* was induced 18-fold under TGF-β1 stimulation, indicating a strong autocrine regulatory loop. The protein expression of a selection of the up-regulated genes was verified using various techniques. TGF-β1 stimulation of CAFs, which secrete low but detectable basal levels of TGF-β1 (Supplementary figure 1D), indeed led to a strong increase of total (latent and active) TGF-β1 protein levels combined with a modest increase in TGF-β1 activity (Figure 2C). Gelatin-zymography analysis revealed strongly increased MMP-2 and MMP-9 levels in the medium of stimulated CAFs compared to non-stimulated cells (Figure 2D). To confirm the role of MMPs in invasion of these CAFs we performed a spheroid invasion assay. CAF spheroids were embedded in a collagen-1 matrix in presence of TGF-β1 or GM6001, a broad-spectrum MMP inhibitor. The data revealed that CAFs invade the collagen matrix, which is slightly induced by TGF-β1 and inhibited by GM6001, confirming the role of MMPs in CAF invasion (Figure 2E).

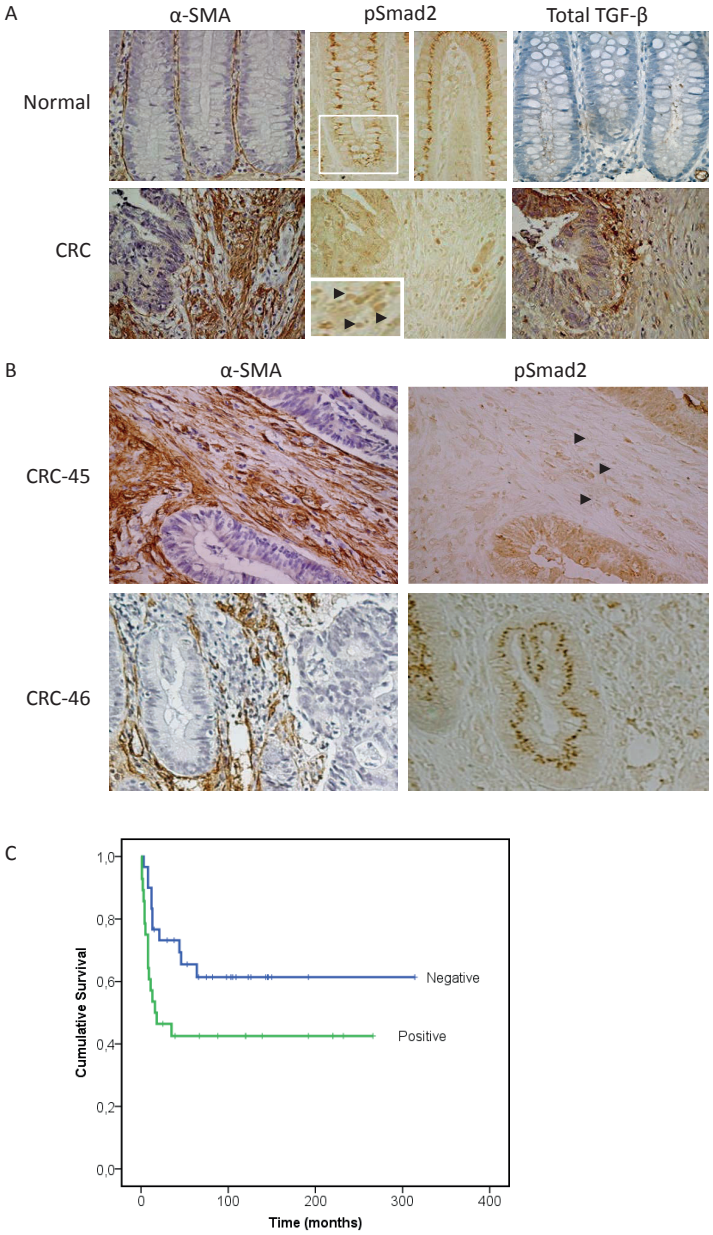


Figure 1 Immunohistochemistry on sequential sections of colorectal cancer (CRC) tissue samples (magnification 200x). Normal colonic mucosa displays a single layer of α-SMA positive myofibroblasts along the crypt axis, which show no nuclear staining for pSmad2, in contrast to epithelial cells showing a gradient in staining from bottom (box) to top of the crypt. Total TGF-β is present in low levels in normal mucosa, whereas it is strongly increased in CRC (**a**). Strongly increased α-SMA expression and a shift from epithelial to mesenchymal nuclear accumulation of pSmad2 (arrow heads) occurs in CRC, although some samples, e.g. CRC-46, still show nuclear accumulation of pSmad2 in malignant cells next to CAFs (**b**). Kaplan-Meier analysis revealed worse survival for patients with positive epithelial pSmad-2 staining (**c**).

Table 2 mRNA expression for MMPs and TGF-β target genes in CRC cell lines and CAFs. Expression of *MMPs* and TGF-β target genes was determined in non-stimulated cells.

Target	HT29	HCT116	SW948	CaCo-2	CAF
MMP-2	++	- (*)	++	+	+++
MMP-3	+	- (*)	++	+/-	+++
MMP-7	+++	+	+	+	++
MMP-9	++	+	++	+	+
MMP-13	+	+/-	++	+	+++
MMP-14	+	+	++	++	+++
MMP-15	++	+	+	++	-
MMP-16	++	++	++	++	++
MMP-17	-	++	++	+	++
MMP-28	-	-	+	-	-
TIMP-1	++	++	++	+++	+++
TIMP-2	++	++	++	++	+++
TIMP-3	+	+	++	++	+++
uPA	++	+	++	+/-	-
PAI-1	++	++	++	+	+++
TGF-β1	+++	+++	+++	+++	+++
Collagen-1	-	-	-	-	+++

– No expression (dCt >20), +/- very low (dCt 15-20), + moderate (dCt 10-15), ++ high (dCt 5-10, +++ very high (dCt <5). All dCt values are expressed versus *GAPDH* as housekeeping gene. CAF = Cancer-associated fibroblast, MMP = Matrix Metalloproteinase, TIMP = Tissue Inhibitor of Matrix Metalloproteinases, uPA = urokinase type Plasminogen Activator, PAI-1 = Plasminogen Activator Inhibitor. (*) = expressed upon TGF-β stimulation.

Characterisation of TGF-β response in CRC cells

To evaluate the variation in TGF-β1 response in CRC cells we first analysed the expression of the TGF-β type II receptor (TβRII) and Smad4 as an important downstream signalling molecule in a panel of CRC cells. HT29 and SW480 cells did not show detectable Smad4 expression, while Lovo cells had very low expression of TβRII (Figure 3A). To further analyse downstream signalling, cells were stimulated with TGF-β1 and pSmad2 was analysed. The data revealed pSmad2 in all cells after TGF-β1 stimulation, except for Lovo cells (Figure 3B). To assess TGF-β signalling more functionally, we used the Smad3/Smad4 transcriptional reporter construct CAGA-luc in all cell lines. LS180, Lovo, SW480, SW948 and CaCo-2 cells showed no induction of luciferase activity after TGF-β1 treatment (data not shown). HT29 cells were partly responsive; only stimulation with concentrations equal to or higher than 5 ng/ml (200 pM) TGF-β1 increased transcriptional activity (Figure 3C). In contrast, HCT116 cells showed dose-dependent induction of luciferase activity up to 5-fold when treated with TGF-β1 (Figure 3D). Addition of ALK-5 kinase inhibitor (SB431542) abolished TGF-β1-induced transcriptional responses. Since TGF-β can inhibit cell proliferation, we analysed this in HT29 and HCT116 cells. These data revealed that cell-proliferation was not altered after 6h (not shown) or 72h of TGF-β1 treatment (MTS proliferation assay, Figure 3E).

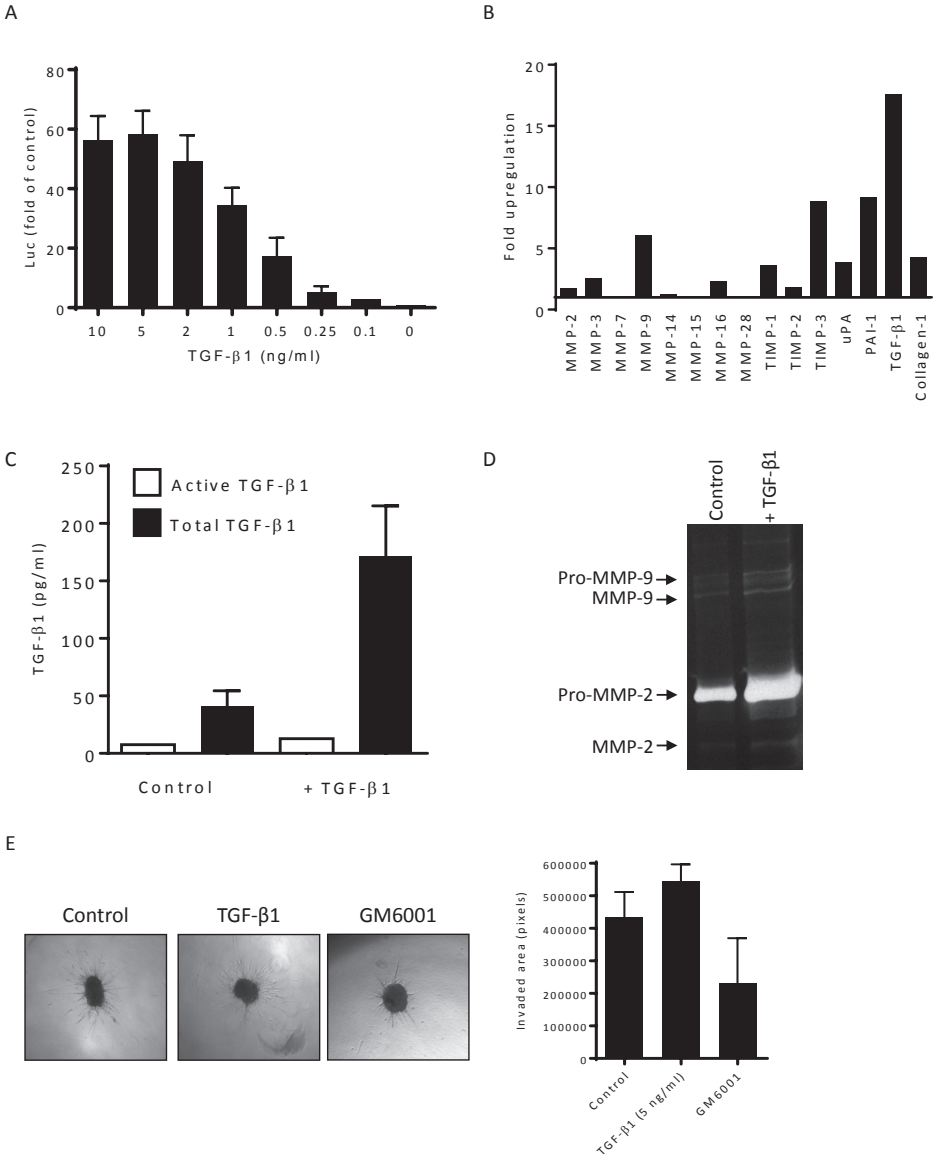


Figure 2 TGF-β-regulated expression of target genes in CAFs. CAFs respond dose-dependently to exogenous TGF-β1 (**a**, n=3-7 independent experiments) and real-time PCR analysis showed upregulation of various proteinases and especially TGF-β mRNA (**b**). TGF-β (**c**, ELISA) and MMP-2 and MMP-9 (**d**, zymogram) secretion into the medium of TGF-β stimulated CAFs is enhanced. For zymogram analysis equal volumes of conditioned medium were loaded. Spheroid invasion assay with CAFs (**e**) show slightly increased invasion of CAFs in a collagen matrix after TGF-β1 treatment and decreased invasion after MMP inhibition (GM6001).

To further characterise these cells we analysed the basal expression levels of *TGF-β1*, *PAI-1,* and invasion related proteinases in HCT116, HT29, SW948 and CaCo-2 cells by qPCR. The data showed high *TGF-β1* expression in all cell lines, intermediate levels of *PAI-1*, and variable expression of *uPA* and *MMPs* (Table 2). As expected, *collagen-1* was not expressed. To analyse TGF-β1-mediated regulation of these genes, HT29 and HCT116 cells were treated with 5 ng/ml (200 pM) TGF-β1 and expression was analysed by QPCR. Figure 4A shows that TGF-β induces *uPA* and *PAI-1* expression in HT29 cells, yet several *MMPs* and *TIMPs* were even stronger up-regulated. In contrast *TGF-β1* mRNA only showed a 2-fold up-regulation. In HCT116 cells a similar pattern was observed (Figure 4B), but the induction of *MMP-2, MMP-13, PAI-1* and *uPA* was much stronger in these cells. However, *TGF-β1* was not changed at all in HCT116 cells after TGF-β stimulation.

Increased proteinase expression is associated with invasiveness of tumour cells via ECM degradation and by processing/release of cytokines. Figure 4C illustrates the effect of TGF-β1 in an invasion assay using HT29 spheroids embedded in a collagen matrix. TGF-β1 treatment clearly induced migration of cells from spheroids into the collagen matrix, eventually leading to distant metastasis-like cell clusters. Unfortunately, the effect on highly responding HCT116 cells could not be evaluated due to poor spheroid formation of these cells.

Latent TGF-β1 activation

Cells secrete TGF-β as a latent complex, which needs processing to become biologically active. Therefore we evaluated whether interactions within the tumour microenvironment could enable activation of the latent TGF-β1 complex. TGF-β responsive HCT116 cells were transfected with the CAGA-luc reporter and incubated with 10 ng/ml (121 pM) recombinant small latent TGF-β1 (SLC), which did not result in increased luciferase activity, implying no activation of the latent complex (Figure 4D). HT29 and HCT116 cells secrete high levels of TGF-β1 (1 and 2 ng/ml respectively, Figure 4E). In addition, HT29 and HCT116 cells secrete minor amounts of TGF-β3, whereas only HCT116 cells secrete TGF-β2 (Figure 4E). To analyse whether these cells secrete large or small latent TGF-β1, conditioned medium (CM) was analysed by western blot for the latency associated protein (LAP) and TGF-β1. Clear bands at ~200 kDa under non-reducing conditions indicate that both HT29 and HCT116 secrete large latent TGF-β1 (Figure 4F). To further evaluate the TGF-β1 response in these cells, HCT116 cells were stimulated with HT29 CM, leading to a minor increase in luciferase activity. In contrast stimulation of HCT116 cells with CAF-CM, containing low levels latent and active TGF-β1 (Figure 2C), induced a 4-fold increase in activity (Figure 4D), almost equal to 5 ng/ml recombinant TGF-β1. These data indicate that the interaction of tumour cells with CAF-derived factors initiates TGF-β signalling, possibly through activation of latent TGF-β1.

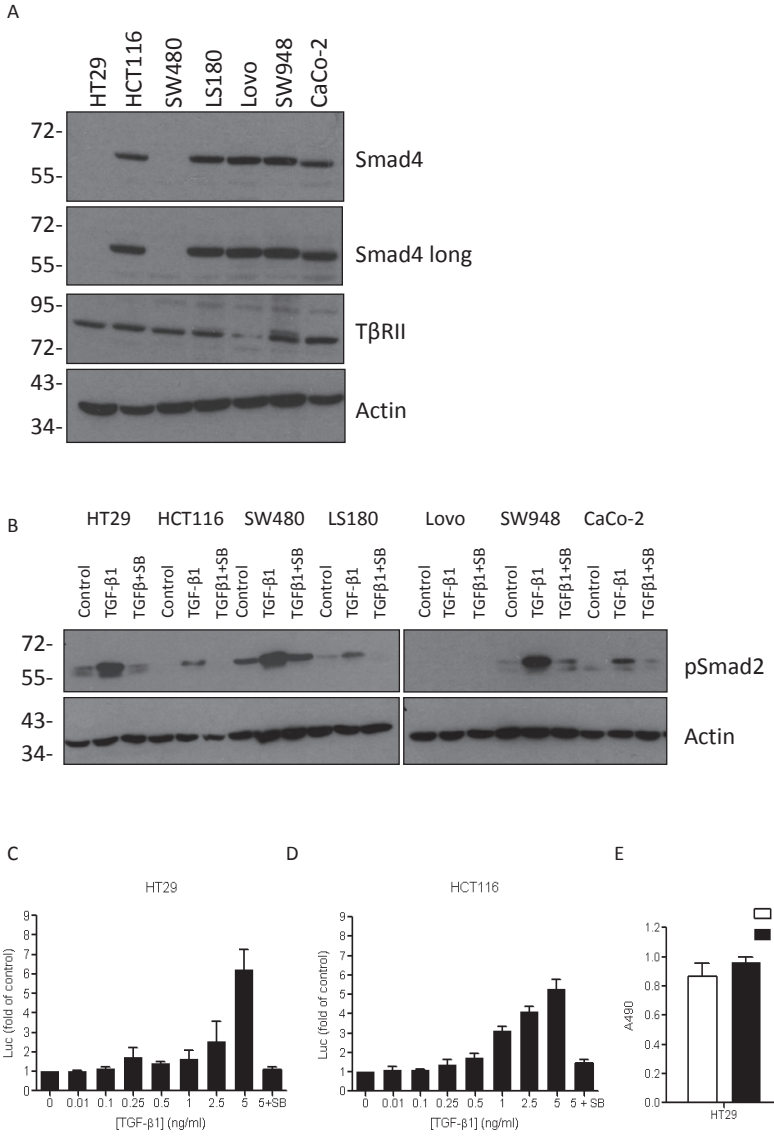


Figure 3 TGF-β response of colorectal cancer cells. Western blot analysis of Smad4 and TβRII expression in colorectal cancer cells (**a**) and downstream signalling after 1h stimulation with TGF-β (**b**, SB= SB431542, an ALK5 inhibitor). HT29 cells only show CAGA response at concentrations of 5 ng/ml or higher (**c**), whereas HCT116 cells dose-dependently respond to TGF-β1 stimulation (**d**). Cell proliferation is not inhibited by TGF-β1 in TGF-β responsive HT29 and HCT116 cells as determined by MTS proliferation assay after 72 hours stimulation (**e**). Data represent 3-7 independent experiments performed in triplicate (mean ± SEM).

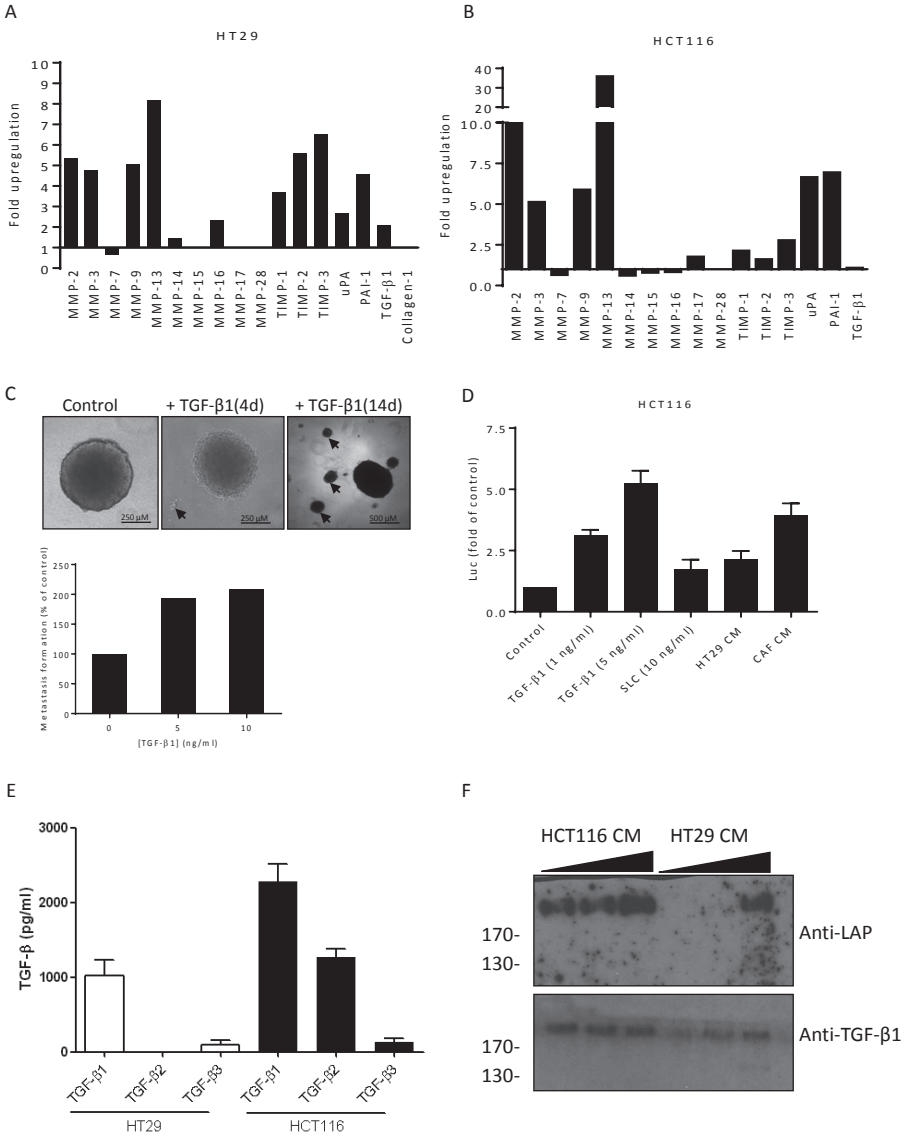


Figure 4 Regulation of proteinases and TGF-β targets genes in tumour cells by TGF-β1. Real-time PCR analysis revealed that HT29 (**a**) and HCT116 (**b**) cells show upregulation of proteinases and TGF-β target genes upon stimulation with 5 ng/ml TGF-β1. 3-dimensional cultures of collagen embedded HT29 spheroids show increased TGF-β1-induced invasive properties and the formation of distant metastasis-like cell clusters (**c**). Experiments using HCT116 cells revealed that these cells are not capable of activating recombinant SLC or HT29 derived LLC. However, strongly increased signalling is observed when combined with CAF-CM (**d**, 3-7 experiments, mean + SEM). HT29 CM contains TGF-β1 and to a lesser extent TGF-β3, whereas HCT116 CM contains higher TGF-β1, TGF-β2 and low levels of TGF-β3 (**e**, ELISA). TGF-β1 is present as large latent TGF-β as shown by western blot analysis under non-reducing conditions (**f**, LAP = latency associated protein).

TGF-β signalling in CAFs and interaction with tumour cells

Since we observed increased TGF-β signalling when HCT116 cells were treated with CAF-CM, we evaluated the response of CAFs after stimulation with tumour cell CM. Stimulation of CAFs with 10 ng/ml (121 pM) SLC or CaCo-2 CM did not increase luciferase activity, whereas stimulation with HT29 CM (~1 ng/ml LLC) revealed a dose dependent 46-fold increase in luciferase activity (Figure 5A). Moreover, treatment with HCT116 CM containing high levels (2 ng/ml) of LLC and only little active TGF-β1 (25-80 pg/ml) led to a dose dependent 71-fold increase compared to control levels. Addition of ALK-5 kinase inhibitor completely abolished the response, confirming that the induction was TGF-β mediated (Figure 5A). Analysis of the CAF medium after incubation with HCT116 CM showed enhanced active TGF-β1 levels (Figure 5B). Furthermore, almost 95% of CAFs treated with HCT116 CM were α-SMA positive, which is comparable to addition of 5 ng/ml TGF-β1, whereas parallel incubations with SLC or HT29 CM showed no change in α-SMA expression (Figure 5C). These data indicate that the interaction of CAFs with HCT116 CM leads to hyperactivation of the TGF-β pathway, involving TGF-β activation and as a consequence α-SMA induction and myofibroblast trans-differentiation.

To evaluate the mechanism of TGF-β activation, CAFs were stimulated with HCT116 CM in the presence of inhibitors of proteases, integrins, or reactive oxygen species, all implicated in latent TGF-β activation *in vitro*. None of the inhibitors, i.e. Aprotinin (plasmin and serine protease inhibitor), GM6001 (broad range MMP-inhibitor), E64 (broad range cystein protease inhibitor), specific inhibitors of MMP-2/-9, MMP-3 and MMP-13 and α2-macroglobulin (BMP-1 and MMP-inhibitor), were able to inhibit the HCT116 CM-induced responses (Figure 5D). To exclude the possible necessity of mutual activating proteolytic cascades, different cocktails of inhibitors were tested. These protease inhibitor cocktails also did not inhibit HCT116 CM induced responses, whereas the ALK-5 kinase inhibitor and neutralizing TGF-β antibody inhibited the signal by more than 90 %.

Hyperactivation of TGF-β signalling in CAFs by HCT116 CM

Since we observed very strong increases in luciferase activity after stimulation of CAFs with HCT116 CM, we evaluated the effects on a major TGF-β target gene, *PAI-1*. Figure 6A-C shows a strong increase of *PAI-1* expression after respectively 6, 12 and 24 hours of TGF-β1 stimulation. Interestingly the induction of *PAI-1* expression by HCT116 CM is higher and lasts longer than stimulation with TGF-β1. Especially after 24h no induction by TGF-β1 is detected anymore, while HCT116 CM still shows 29-fold induction of *PAI-1* mRNA. We further evaluated the underlying mechanism by western blot. Analysis of downstream pSmad2 and pSmad3 revealed increased phosphorylation after stimulation with HCT116 CM compared to TGF-β1 (Figure 6D). Similarly PAI-1 protein levels after 6h were higher in HCT116 CM compared to TGF-β1 stimulated cells. Finally stimulation of CAFs with HCT116 CM led to earlier and prolonged (up to 360 min) pSmad2 levels (Figure 6E) followed by higher PAI-1 protein expression. To examine whether the expression of invasion related proteinases is also enhanced upon HCT116 CM stimulation, we analysed expression of

MMP-2, MMP-3 and *MMP-14*. mRNA levels of *MMP-2* were similar in HCT116 CM and TGF-β1 treated cells whereas *MMP-3* and *MMP-14* were two fold stronger induced by HCT116 CM than by TGF-β1. (Figure 6F). Furthermore *TGF-β1* mRNA levels were also strongly up-regulated after HCT116 CM stimulation. Stimulation with HT29 CM showed a similar trend, although less pronounced as HCT116 CM, confirming the CAGA-luc reporter data (Figure 5A). These data indicate hyperactivated TGF-β signalling accompanied by increased expression of TGF-β regulated genes in CAFs as a result of interaction with epithelial tumour cell-derived soluble factors.

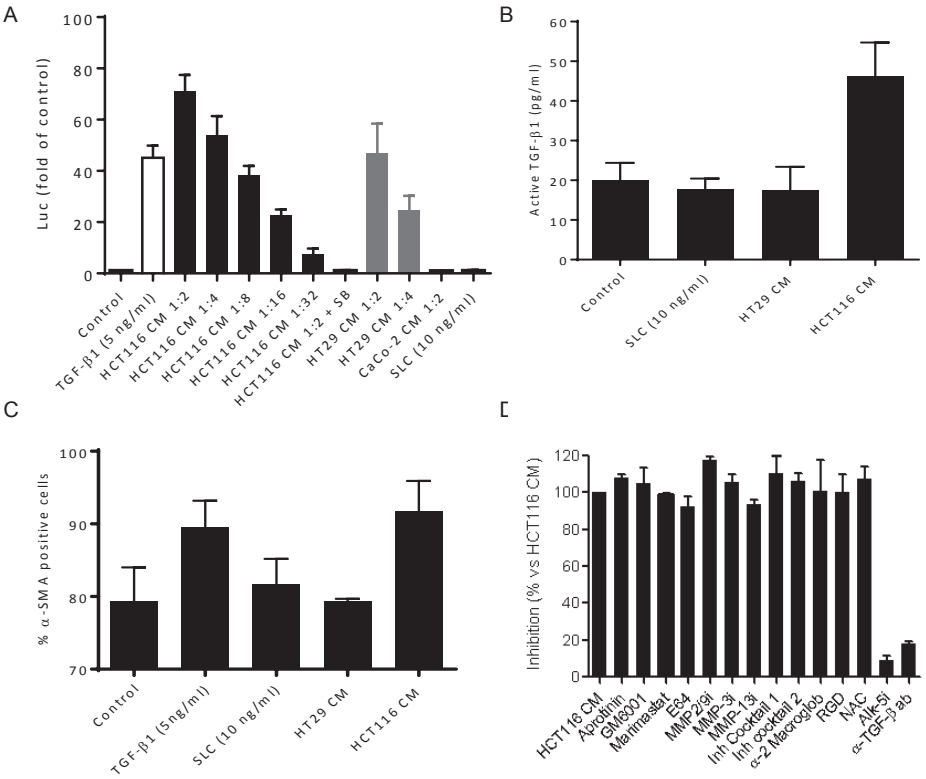
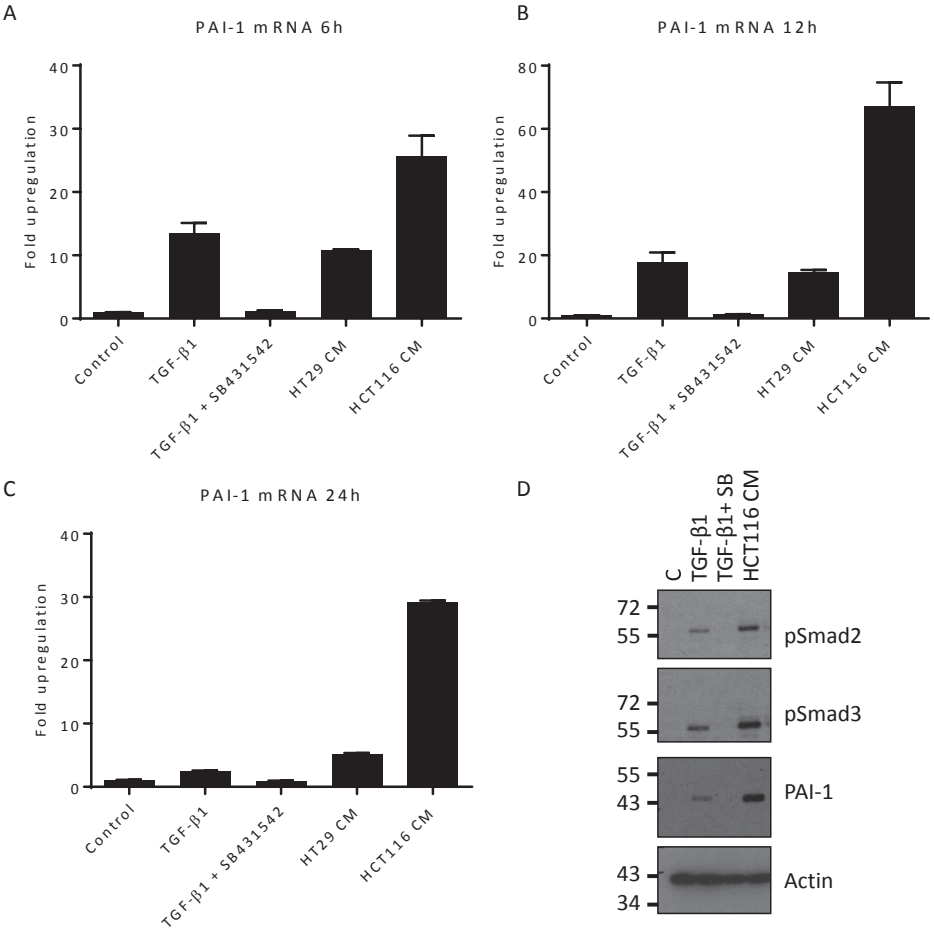


Figure 5 Co-culture of fibroblasts with HCT116 CM or to a lesser extent HT29 CM leads to increased TGF-β signalling (**a**), elevated active TGF-β1 levels in medium after stimulation (**b**) and trans-differentiation into myofibroblasts as shown by increased α-SMA levels on cytospins (**c**). Induction of TGF-β response cannot be inhibited by addition of proteinase inhibitors or inhibitor cocktails, but is inhibited by ALK-5 inhibitor (SB431542) or neutralising pan-TGF-β antibody (**d**). Data represent percentage inhibition versus HCT116 CM (set to 100%), 3-7 independent experiments, mean + SEM.


A
PAI-1 mRNA 6h
Fold upregulation
40
30
20
10
0
Control
TGF-β1
TGF-β1 + SB431542
HT29 CM
HCT116 CM
B
PAI-1 mRNA 12h
Fold upregulation
80
60
40
20
0
Control
TGF-β1
TGF-β1 + SB431542
HT29 CM
HCT116 CM
C
PAI-1 mRNA 24h
Fold upregulation
40
30
20
10
0
Control
TGF-β1
TGF-β1 + SB431542
HT29 CM
HCT116 CM
D
C
TGF-β1
TGF-β1+ SB
HCT116 CM
72
55
pSmad2
72
55
pSmad3
55
43
PAI-1
43
34
Actin
E
t=0
t=15
t=60
t=180
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C C T H C T H C T H C T H
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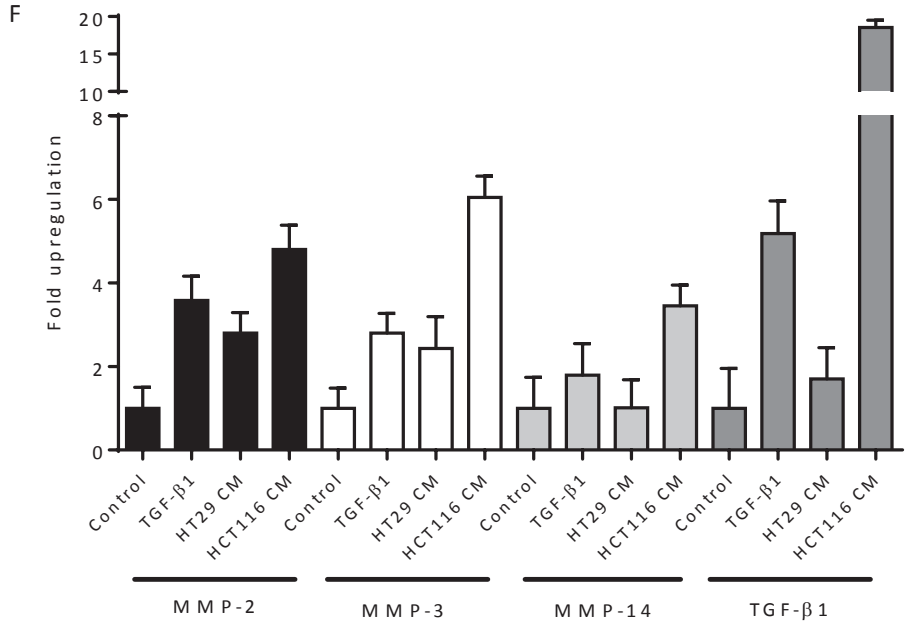


Figure 6 Hyper-activation of TGF-β signalling in CAFs. Expression of the TGF-β target gene *PAI-1* was analysed in CAFs 6h (**a**), 12h (**b**) and 24h (**c**) after stimulation with TGF-β1, HT29 CM or HCT116 CM. Induction of expression by HCT116 CM is stronger and prolonged compared to TGF-β1 stimulation. Enhanced Smad2 and Smad3 phosphorylation as well as PAI-1 expression 6 hours after stimulation with HCT116 CM (**d**). Analysis of Smad-2 phosphorylation in time revealed earlier and prolonged phosphorylation after stimulation with HCT116 CM (**e,** c=control, T = TGF-β1, H = HCT116 CM). Expression of TGF-β regulated, invasion related MMPs revealed stronger increase in HCT116 CM stimulated compared to TGF-β1 stimulated CAFs. The induction of TGF-β1 expression is even more pronounced (**f**).

Discussion

The present study shows that TGF-β signalling in CRC shifted from mainly epithelial TGF-β signalling to signalling in CAFs. In addition, we have shown that the interaction between CAFs and epithelial tumour cells leads to the hyperactivation of TGF-β signalling in CAFs, accompanied by increased expression of invasion-related proteinases and elevated TGF-β secretion.

Although most cancers originate from epithelial cells, the tumour stroma might actively participate in the progression of carcinomas, as was illustrated by the prognostic relevance of stroma quantity for the survival of CRC patients (19). Within the tumour microenvironment especially CAFs are associated with auto- and paracrine signalling (1;6), hereby influencing epithelial cell proliferation and enhancing the tumours' invasive and metastatic potential (20). Interactions between cancer cells and CAFs can occur through direct cell-cell contact via for instance extracellular matrix metalloproteinases inducer (EMMPRIN), a potent inducer of the myofibroblast phenotype (21). However the

majority of interactions are mediated by soluble factors. Amongst the fibroblast-derived soluble factors affecting epithelial cancer cells are scatter factor/hepatocyte growth factor (SF/HGF) (22;23), insulin like growth factor, TGF-β1 and several others (24;25). TGF-β1 is implicated in activation of fibroblasts leading to the generation of CAFs (26;27). Our immunohistochemistry data confirmed the shift in TGF-β signalling from epithelial cells to CRC CAFs. In normal mucosa (myo-) fibroblasts showed hardly any nuclear pSmad2 staining, indicating low TGF-β signalling in the normal stroma. In CRC however, signalling in most of the epithelial cancer cells was lost, which corresponds to previous studies showing that in later stages epithelial cells become refractory to the TGF-β growth inhibiting properties (12;28;29). *In vitro* experiments have shown that the majority of CRC epithelial tumour cell lines are not responsive to TGF-β, a phenomenon caused by mutations in the TβRII, Smad4 or other pathway components (30;31). Our data show that even CRC cells with a functional TGF-β pathway are not growth inhibited by TGF-β1, but instead respond through increased expression of invasion related proteinases. This is further confirmed by the observation that CRC patients with present epithelial TGF-β signalling show worse disease-free survival compared with patients with absent TGF-β signalling.

With regard to CAFs we observed an enrichment of pSmad2 positive population in CRC indicating active TGF-β signalling in these cells, probably due to increased levels of TGF-β1 in the tumour microenvironment. *In vitro* CAFs responded robustly to (tumour derived)-TGF-β1 by trans-differentiation into α-SMA-expressing myofibroblasts and concomitant up-regulation of target genes *PAI-1*, *collagen-1*, *MMPs* and particularly *TGF-β1*. Up-regulation of latent growth factors is a common phenomenon in many cancer types, but the subsequent activation is even more crucial for their effects (32;33). For TGF-β1 we have shown previously that although latent TGF-β1 is already up-regulated in pre-malignant adenomas, increased TGF-β1 activity is only observed in CRC (34), suggesting that over-activation and not over-expression is characteristic of malignant progression. In the present study we showed that CAFs were not effective in releasing TGF-β1 from the SLC. However treatment with tumour cell-derived CM induced TGF-β signalling in CAFs and expression of target genes, including invasion related proteinases, to a higher extent than stimulation with recombinant TGF-β1. Earlier studies already showed that co-culture of dermal fibroblasts with keratinocytes increased myofibroblast numbers (35) and that breast cancer cells in co-culture with fibroblasts are also able to induce the myofibroblast phenotype via direct cell-cell contact (36). Our data indicate hyperactivation of TGF-β signalling in CAFs in a TGF-β1 dependent manner with collaboration of other soluble factors, secreted by CRC cells, leading to CAFs activation.

TGF-β activation occurs via proteolytic processing of the LLC by MMPs, plasmin or BMP1, followed by a conformational change generated by integrins (13;15;16;37-39). Another mechanism involves TGF-β activation by reactive oxygen species (ROS) (40). Since we observed increased TGF-β signalling after incubation with tumour cell-derived CM

containing mainly latent TGF-β1 and this effect was accompanied by increased active TGF-β1 levels, we also evaluated the potential activation mechanism of TGF-β1. No inhibition of TGF-β signalling after addition of protease inhibitors, an RGD peptide blocking integrins, or ROS inhibitor n-acetylcysteine was observed. To exclude the possibility that cascades of proteolytic activation of pro-proteinases would be required cocktails of inhibitors were used, which also did not show any effect. Other factors secreted by tumour cells might also influence TGF-β effects on myofibroblast differentiation, as has been shown for interleukin-1, which can inhibit TGF-β induced myofibroblast differentiation (35). However, incubation with an ALK-5 kinase inhibitor or neutralising pan-TGF-β antibody completely abolished the induction of target genes and reporter activity indicating a strong TGF-β dependency.

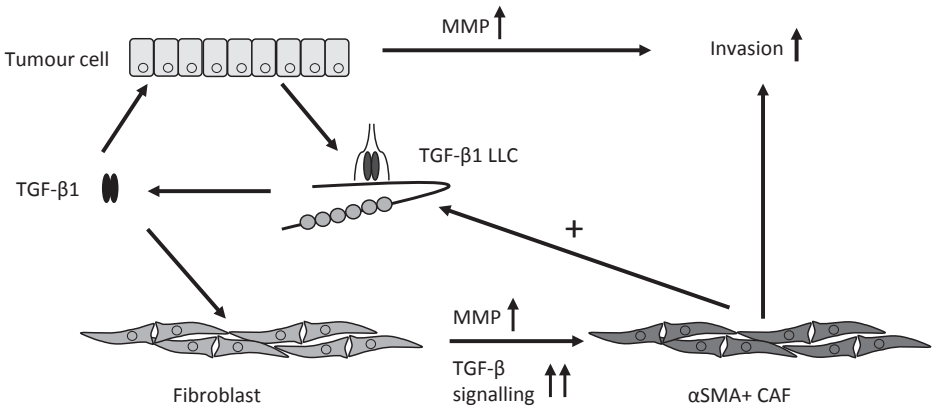


Figure 7 Proposed model for the interaction between tumour cells and CAFs in the tumour microenvironment. The interaction between tumour cell-derived soluble factors induced hyperactivation of TGF-β signalling in CAFs in a TGF-β dependent manner, leading to enhancement of MMP and TGF-β1 expression. These result in increased invasive behaviour and enhanced TGF-β1 secretion, thereby creating a cancer promoting feedback loop in the tumour microenvironment.

In conclusion, we have shown that the soluble, non-cell-contact dependent interaction between epithelial tumour cells and CAFs increases proteinase expression and thereby tumour progression. We propose that tumour cells during cancer progression lose the anti-proliferative response to TGF-β and gain pro-invasive properties through increased proteinase production. Epithelial tumour cells produce high amounts of TGF-β1 LLC, which hyperactivates TGF-β signalling in fibroblasts leading to their trans-differentiation into CAFs. CAFs in turn show strongly increased production of proteinases and TGF-β1 creating a cancer progressing feedback loop (Figure 7). These data further emphasize the role of tumour-stroma interactions, in particular myofibroblasts, and validate further exploration of the tumour stroma, including TGF-β as potential therapeutic target (3;25;41).

Conflict of interest

All authors declare no conflict of interest.

Acknowledgements

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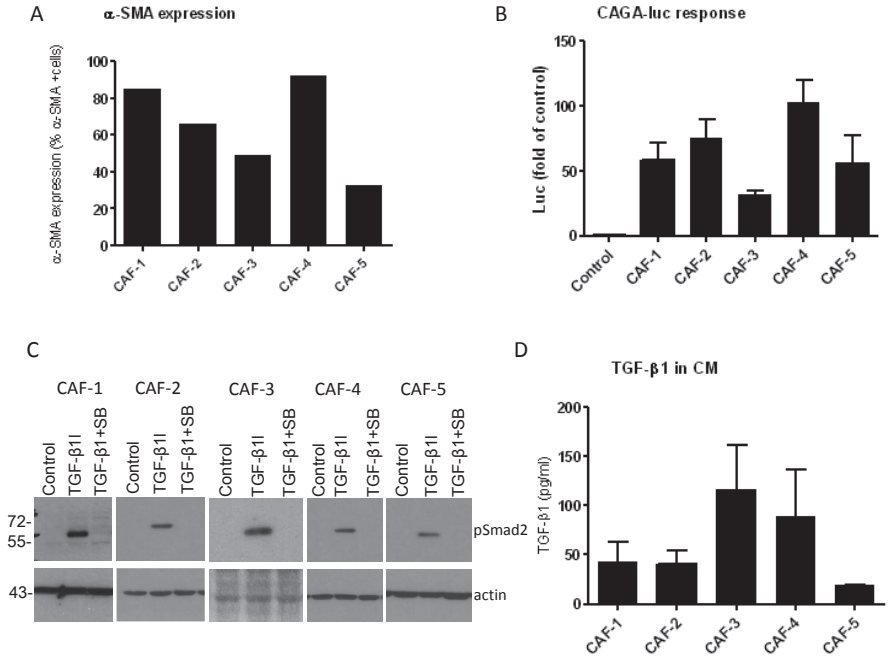
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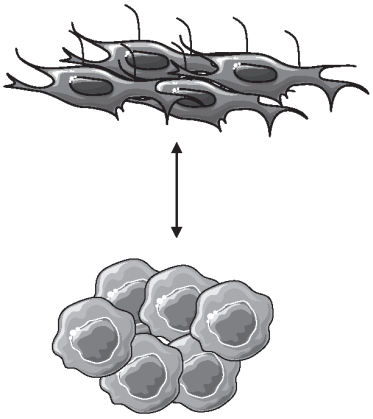
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Supplementary data



Supplementary figure 1. Characterization of colorectal cancer CAFs. CAFs were isolated from colorectal cancer specimens and analyzed for α-smooth muscle actin (α-SMA) expression (**a**). Next the response to exogenous added TGF-β1 by the CAGA-luc reporter was evaluated (**b**). Additionally all CAFs show similar phosphorylation of Smad-2 upon TGF-β1 stimulation which is blocked after addition of TβRI kinase inhibitor SB431542 (**c**). Finally TGF-β1 secretion by all different CAFs was analyzed in 4 day conditioned medium by ELISA (**d**).



Chapter 7

Endoglin expression on cancer-associated fibroblasts regulates fibroblast invasion and plays an important role in colorectal cancer metastasis

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Manuscript in preparation

Abstract

Cancer-associated fibroblasts (CAFs) are the major component of the tumor microenvironment in solid tumors, including colorectal cancer (CRC). CAFs play an important role in all phases of carcinogenesis, including tumor progression and metastasis. Endoglin is a co-receptor for members of the transforming growth factor-β family and is highly expressed on activated endothelial cells, but also on other cells. In this study we showed that endoglin is expressed on CAFs specifically at the invasive borders of colorectal tumors, suggesting a role in tumor invasion. Next, we explored the role of endoglin on CAFs in CRC progression and metastasis. Endoglin expressing CAFs were detected in the primary tumor and in lymph node and liver metastases of stage-IV patients. Moreover, in stage-II CRC patients, CAF-specific endoglin expression at the invasive front was related to metastasis-free survival. *In vitro* experiments revealed that endoglin is indispensable for bone morphogenetic protein (BMP)-9-induced signaling and cell survival in fibroblasts. Migration assays showed that fibroblast invasion is, at least partly, dependent on endoglin expression *in vitro*. In a zebrafish model for CRC, we could show that endoglin expression on fibroblasts enhanced tumor cell infiltration into the liver. Finally, we used a mouse model for experimental CRC metastasis to show that endoglin targeting with the neutralizing antibody TRC105 decreased metastatic spread to the liver. These results show that endoglin expressing CAFs contribute to CRC metastasis. Furthermore, this study identifies an additional mechanism of action for endoglin targeted therapy beyond targeting of the endothelium, explaining beneficial effects seen during the clinical evaluation of TRC105.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide (*1*). Although improvements in therapeutic strategies have decreased mortality rates in the past decades, 8.5% of all cancer deaths is still ascribed to CRC. 90% of cancer patients die because of metastatic spread to distant organs. Therefore, patients identified to be at risk to develop metastatic disease (stage III/IV) receive adjuvant chemo- or radiation therapy (*2*). Early stage patients are not adjuvantly treated, but still a significant proportion develops metastatic disease. This underlines that current patient selection is not sufficiently accurate, which might be improved by a better understanding of the mechanism underlying tumor metastasis.

Next to malignant epithelial cells, solid cancers consist of the tumor stroma, which can make up to 50% of the tumor mass. Tumor stroma content has been shown to be predictive for survival of CRC patients (*3*). The tumor stroma, or tumor microenvironment (TME), is composed of vascular endothelial cells, immune cells and cancer-associated fibroblasts (CAFs) (*4*). CAFs are the major component of the TME, and interact with all other cells, including epithelial tumor cells, both via direct cell-cell contacts and via CAF-derived cytokines (*5, 6*). This leads to tumor progression and eventually metastasis. Therefore, CAFs are considered a potential target in developing novel cancer therapies.

Although the origin of CAFs is under debate, resident fibroblasts are considered to be one of the main cells of origin (*7*). Transforming growth factor-β (TGF-β) is a cytokine capable of transdifferentiating resident fibroblasts into CAFs (*8, 9*), indicated by increased expression of α-smooth muscle actin (αSMA) and fibroblast activation protein (FAP) (*10*).

Endoglin is a co-receptor for TGF-β family members, highly expressed on the surface of activated endothelial cells and is indispensable during embryogenesis (*11-13*). In endothelial cells, binding of the endoglin ligands TGF-β and bone morphogenetic protein-9 (BMP-9) induces downstream Smad1 phosphorylation, resulting in angiogenesis by increased proliferation and migration of endothelial cells (*14*). Furthermore, endoglin microvessel density was reported to correlate with tumor progression and metastases in CRC (*15, 16*). The endoglin neutralizing antibody TRC105 binds human endoglin with high affinity, prevents BMP-9 binding to endothelial cells and induces an antibody-dependent cell-mediated cytotoxicity response (*17, 18*). Consequently, TRC105 is currently under clinical development as anti-angiogenic therapy.

In this study we have investigated the role of endoglin expression on CAFs. We showed that CAFs, specifically at the invasive borders of colorectal tumors, express endoglin. Additionally, endoglin is also expressed on CAFs in metastatic lesions. Next, regulation of endoglin expression on human CAFs was determined. Using *in vitro* invasion assays, we show that endoglin is involved in CAF migration and invasion. Additional evidence was shown in a novel zebrafish model for CRC. Finally, in a mouse model for experimental CRC metastasis, we showed that neutralizing endoglin with TRC105 decreased metastatic spread in mice co-injected with tumor cells and CAFs. Taken together, our data suggest

an additional working mechanism for endoglin targeted therapy, besides targeting the endothelium.

Materials and methods

Patient samples

Paraffin-embedded tissue samples were obtained from the Department of Surgery, Leiden University Medical Center. One cohort consisted of 104 stage-II and 94 stage-III CRC patients who underwent surgery without pre-treatment. Another cohort consisted of respectively resection specimens of primary tumor, lymph node and liver metastases from 31 individual patients. Patient characteristics and follow-up were recorded by the Department of Surgery. Samples were used according to the guidelines of the Medical Ethical Committee of the Leiden University Medical Center.

Tissue analysis

Immunohistochemical stainings were performed as described before (*19*), using antibodies against CD31 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), endoglin (R&D Systems, Abington, UK), pan-cytokeratin (Dako, Glostrup, Denmark) and αSMA (Progen, Heidelberg, Germany). Endoglin expression on CAFs was scored on a scale of 1 to 4 in a blinded manner by two independent observers. Score 1; ≤10% CAFs positive, score 2; 10-25% CAFs positive, score 3; 25-50% CAFs positive, score 4; ≥50% CAFs positive. Manual scores were confirmed using ImageJ (National Institute of Health), by excluding vessel-specific endoglin expression and quantification of CAF-specific endoglin. Pictures were obtained using a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). For immunofluorescence, paraffin embedded sections were deparaffinized and rehydrated through graded alcohol. Antigen retrieval was performed by boiling in citrate solution (Unmasking solution, Vector Laboratories, Burlingame, CA, USA) for 10 minutes. Sections were blocked by incubation in 2% horse serum for 1 hour, at room temperature. After rinsing in phosphate-buffered saline (PBS), slides were incubated with mouse anti-αSMA (Progen) and goat anti-endoglin (R&D) antibodies overnight at 4°C. After washing in PBS, sections were incubated with donkey anti-goat Alexa 888 and donkey anti-mouse Alexa 555 (Waltham, MA, USA) for 90 minutes, at room temperature. After washing in PBS, slides were incubated with TO-PRO nuclear counter stain (ThermoFisher) for 5 minutes and mounted in Prolong DAPI (ThermoFisher). Microscopic pictures were obtained using a Leica SP8 confocal scanning laser microscope (Leica Microsystems B.V., Son, The Netherlands). Quantitative PCR analyses were performed as described before (*6*). Primer sequences are described in supplementary table S1.

Cell culture and signaling assays

Primary human colorectal fibroblasts and the mouse CRC cell line MC38 (*20*) were cultured in DMEM/F12, supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 50 µg/mL

gentamycin, 100 IU/mL penicillin and 100 µg/mL streptomycin (all ThermoFisher). Human embryonic kidney 293 (HEK293T) cells were maintained in DMEM, supplemented with 10% FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin (all ThermoFischer). Human CAFs were isolated from non-necrotic parts of the tumors and normal fibroblasts (NFs) from adjacent healthy tissue as described before (*6*). The tissue was cultured in DMEM/F12, supplemented with 10% FCS, 10 mM HEPES, 50 µg/mL gentamycin, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL Fungizone (all ThermoFisher). After 7-10 days, fibroblast-like cell outgrowth was observed. Murine embryonic fibroblasts (MEFs) were obtained from E12.5 embryos as described before (*21*) from a mouse strain in which exons 5 and 6 of the endoglin gene are flanked by LoxP sites (*22*). MEFs were maintained in DMEM, supplemented with 10% FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin (all ThermoFischer). All cell cultures were monthly tested for mycoplasma contamination.
Constructs expressing human endoglin (*23*), Cre recombinase (pLV.mPGK.iCRE.IRES.PuroR, kindly provided by Dr. M. Goncalves, Leiden University Medical Center) or endoglin short hairpin RNA (shRNA, Sigma Mission shRNA library, constructs SHC001, TRCN0000083138, TRCN0000083139, TRCN0000083140, TRCN0000083141 and TRCN0000083142) were delivered by lentiviral transduction using polybrene (4 µg/mL, Sigma Aldrich) to 80% confluent fibroblasts. After 48 hours, transduced cells were selected by 1.5 µg/mL puromycin treatment. HEK293T cells were grown to 80% confluency and transfected with endoglin expressing plasmids using 1 mg/mL polyethylenimine (PEI; Polysciences Inc., Warrington, PA, USA).
For signaling assays, fibroblasts were seeded in six-well plates. Upon 90% confluency, cells were serum starved overnight in medium without antibodies or containing either 40 µg/mL TRC105 (Tracon Pharmaceuticals, San Diego, CA, USA) or 40 µg/mL human IgG (BioXCell, West Lebanon, NH, USA). Next day, cells were stimulated with either 5 ng/mL TGF-β3 (*24*), 0.1 ng/mL BMP-9 (R&D Systems) or 100 ng/mL BMP-6 (PeproTech, London, UK) for one hour. Cells were lysed in RIPA buffer, protein content was determined and western blot analysis was performed as described before (*25*). Membranes were incubated overnight with primary antibodies against endoglin (R&D Systems), phosphorylated Smad1 or phosphorylated Smad2 (Cell Signaling Technologies, Danvers, MA, USA). Blots were stripped and reprobed with mouse anti-GAPDH antibody (Millipore, Amsterdam, The Netherlands) as loading control. Blots were developed using the Bio-Rad ChemiDoc Touch Imaging System (Bio-Rad, Veenendaal, The Netherlands).

Invasion assays

1000 HEK293T cells or 2500 CAFs were seeded on 0.6% agarose coated 96-well plates and left to form spheroids for two days. After two days, spheroids were collected and embedded in 1 mg/mL collagen-I matrix (Advanced BioMatrix, San Diego, CA, USA) containing 10% FCS. At 0, 24 and 48 hours, pictures were taken using a Zeiss Axiovert 200M microscope (Carl Zeiss BV, Sliedrecht, The Netherlands). Quantification of the invaded area for HEK293T cells was performed using Adobe Photoshop software.

For transwell invasion assays, the upper surface of 8.0 µm pore size inserts (Greiner Bio-One, Alphen aan den Rijn, The Netherlands) was coated with 200 µg/mL collagen-I in culture medium containing 0.5% FCS. The lower well of the transwell system was filled with medium containing 10% FCS. $5*10^5$ fibroblasts were seeded on top of the coated insert in medium containing 0.5% FCS and left to invade for 24 hours. After 24 hours, invaded cells were fixed to the membrane using 4% paraformaldehyde and stained with crystal violet. Using an Olympus BX51TF microscope (Olympus Life Science Solutions, Zoeterwoude, The Netherlands), five pictures per insert were obtained at 2x magnification. Cell invasion was quantified in at least three independent experiments using triplicates, either by counting cells or by calculating the percentage area positive for crystal violet staining, using Image J software (National Institutes of Health, USA).

Zebrafish

Zebrafish were maintained according to standard methods approved by the animal welfare committee of the Leiden University. 2-day old Tg(fli1:GFP) (*26*) dechorionated zebrafish embryos were injected with 400 cells in the heart cavity: either 400 dTomato-labeled MC38 cells or 200 dTomato-labeled MC38 in combination with 200 MEFs. After injection, embryos were maintained at 33°C. Injected embryos were sedated with 0.003% tricaine (Sigma, Zwijndrecht, The Netherlands) and transferred to a 3% agarose coated dish for imaging. Fluorescent image acquisition was accomplished using a Leica SP5 STED confocal microscope (Leica Microsystems B.V., Son, The Netherlands). Confocal stacks were processed using Leica software.

Experimental metastasis model

All animal experiments were approved by the Dutch animal ethics committee. 20-weeks old Crl:CD-1Fox1nu male mice (Charles River, L'Arbresie Cedex, France) were anesthetized using isoflurane. $5*10^5$ HT29 cells selected for liver metastasizing potential and expressing firefly luciferase were injected in the spleen, either alone or in combination with 10^5 CAFs, dissolved in 50 µL medium. 24 hours before injection, CAFs were pre-treated with either 40 µg/mL TRC105 or 40 µg/mL human IgG, as a control. Mice were treated twice weekly, starting 2 days after injection, with either 15 mg/kg TRC105 or 15 mg/kg human IgG, intraperitoneally. Metastatic spread was monitored twice weekly using bioluminescent imaging on the IVIS Lumina-II (Caliper Life Sciences, Hopkinton, MA, USA). Three weeks after injections, mice were sacrificed and blood and tissue samples were collected. To assess metastases, livers were subjected to *ex vivo* bioluminescent imaging.

Statistical analysis

Differences between two groups were calculated using Students'*t*-test. Survival curves were generated using Kaplan-Meier analysis and Log rank test. Differences in bioluminescent signals over time in mice were calculated using 2way ANOVA analysis, complemented with Bonferroni posttests. P-values of ≤0.05 were considered statistically significant.

Results

Endoglin expression on CAFs correlates with metastasis-free survival in stage-II CRC

To investigate the endoglin expression pattern in CRC, we stained sequential slides of CRC tissues for cytokeratin (epithelium), CD31 (endothelial cells), αSMA (CAFs) and endoglin. As expected and previously described, endoglin is highly expressed by angiogenic endothelial cells, as shown by the overlap between CD31 and endoglin staining (Fig. 1A, white arrow heads). Endoglin microvessel density is therefore clinically used as a predictive marker in various solid tumors (*27*). However, we also observed endoglin expression on CAFs at the invasive borders, as indicated by co-staining of endoglin and αSMA (Fig. 1A, black arrow heads). Co-localization of αSMA and endoglin was additionally shown using immunofluorescence (Fig. 1B and supplementary Fig. S1). Fibroblasts in adjacent normal colonic tissue or CAFs in the core the tumor did not express endoglin (Fig. 1C, black arrow heads). The specific localization of endoglin expressing CAFs at the invasive border (Fig. 1C, lower panels) and their absence in the tumor core (Fig. 1C, middle panels) implies that endoglin on CAFs plays a role in CRC invasion and metastasis.

To explore this hypothesis, we stained primary tumors, lymph node and liver metastases from the same patients for cytokeratin, αSMA and endoglin (Fig. 2A). In this cohort of 31 patients endoglin expression was present on CAFs at the invasive border of primary tumors, but staining intensity was remarkably higher on CAFs in both lymph node and liver metastases, underscoring the involvement of endoglin expressing CAFs in the formation of CRC metastases (Fig. 2A).

To assess whether endoglin expression on CAFs could be predictive for the development of metastatic disease, we stained a cohort of stage-II and stage-III CRC patient tissues for αSMA and endoglin and assessed the relation between endoglin expression and metastasis-free survival. Endoglin expression on blood vessels was not included in our analysis. In the stage-II cohort, high endoglin expression significantly correlated to worse metastasis-free patient survival (Fig. 2B). In contrast, in stage-III CRC patients, no significant correlation between either overall survival or time to distant metastases and CAF-specific endoglin expression was observed (supplementary Fig. S2), although the overall staining intensity for endoglin on CAFs was higher. Since stage-III patients already present with lymph node metastasis, this suggests that endoglin expressing CAFs could play a role in CRC invasion and metastasis in early phases of tumor progression. Taken together, these data suggest a potential role for endoglin on CAFs in regulating the metastatic potential of early stage CRC.

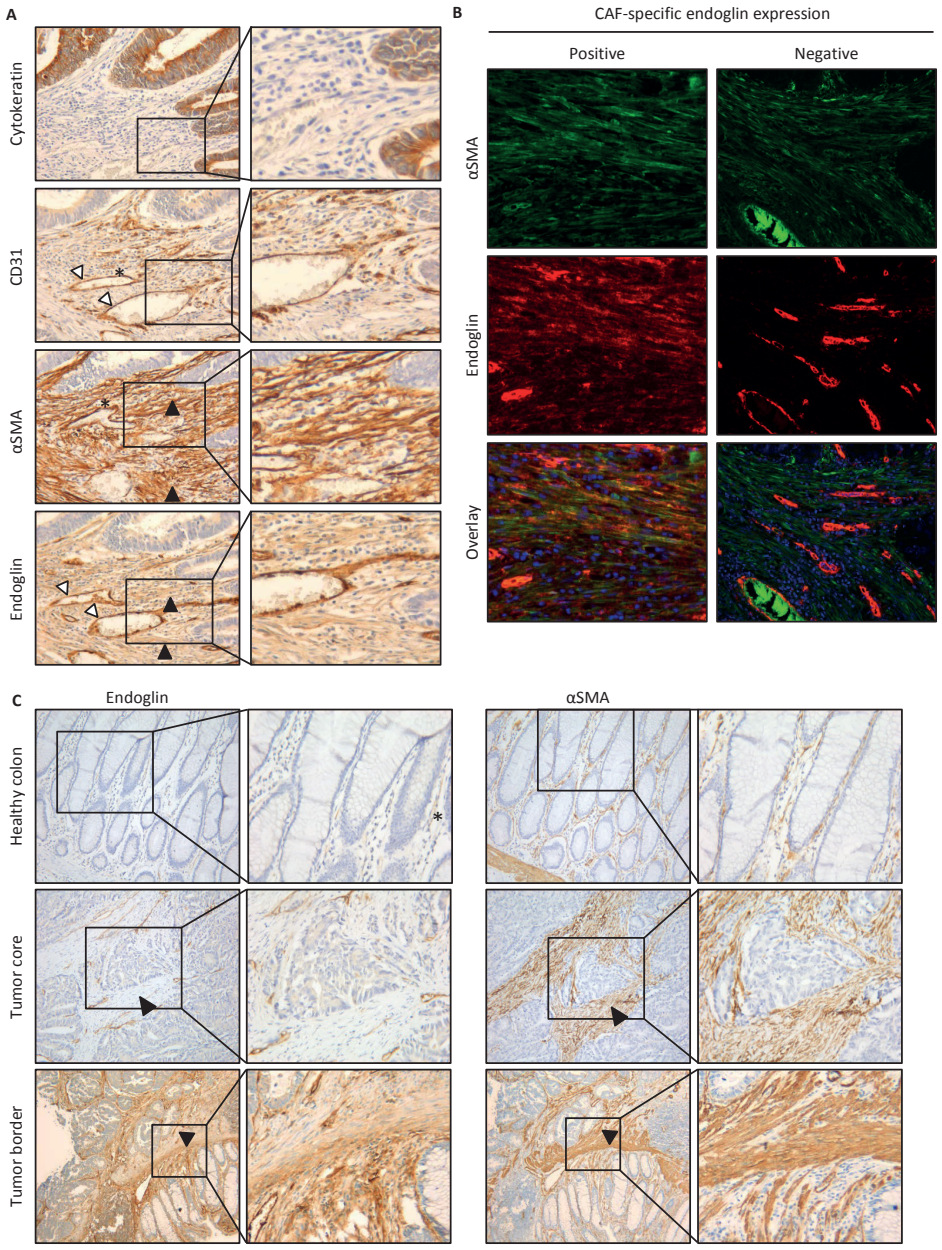


Figure 1. CAF-specific endoglin expression in CRC. A. CRC tissue was stained for cytokeratin, CD31, αSMA and endoglin. Overlap between endoglin/CD31 (white arrow heads), indicating high endoglin expression on the vasculature, and endoglin/αSMA (black arrow heads), showing endoglin expression on CAFs, was observed. Asterisks indicate αSMA expressing smooth muscle cells, surrounding the vasculature. B. Endoglin staining (red) in CRC patient tissue showed high expression on blood vessels. Overlap between endoglin and αSMA (green) indicates endoglin expression in CAFs. Tissue from another CRC patient did not show endoglin expression on CAFs, although highly expression on endothelium. C. Immunohistochemical staining for expression of endoglin and αSMA on healthy tissue, in the tumor core and invasive border from the same CRC patient.

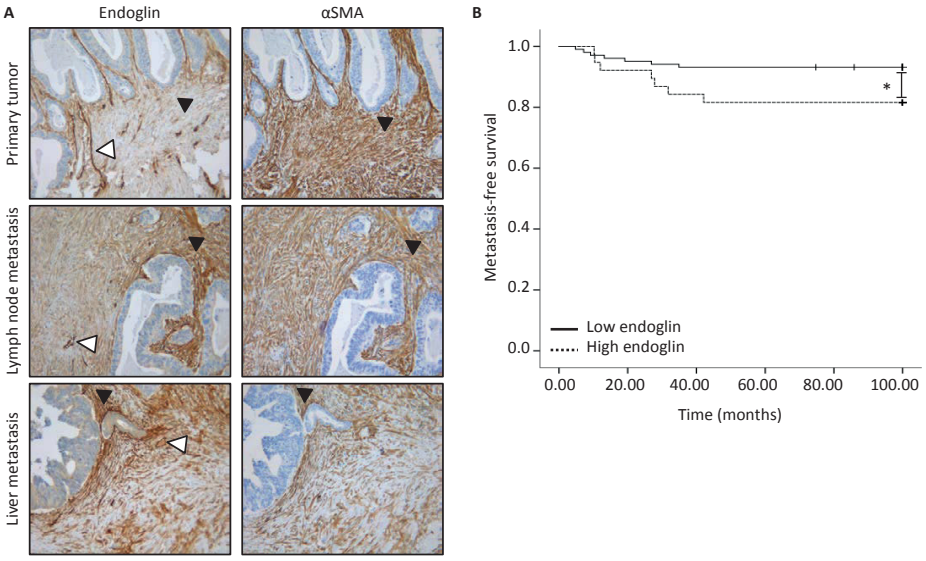


Figure 2. Endoglin expression on CAFs at invasive border correlates to metastasis-free survival in stage-II CRC. A. Primary tumor, lymph node and liver metastases from the same stage-IV CRC patient show endoglin expressing CAFs (black arrow heads). Endoglin expression on vasculature is indicated with white arrow heads. B. Endoglin expression on CAFs at the invasive border of colorectal tumors correlates to metastasis-free survival in stage-II CRC patients (n=140) *p≤0.05.

Receptor expression profiles do not differ between normal fibroblasts and CAFs *in vitro*

Since endoglin is expressed on CAFs and might play a role in CRC metastasis, we set out to assess its expression on fibroblasts. For this we isolated CAFs from CRC tissues and normal fibroblasts (NF) from adjacent normal appearing mucosa (>10 cm from the primary tumor) from four different patients. Although we have observed that NFs appear to get activated upon culturing and start to express certain CAF markers, like αSMA, we set out to determine differential gene expression patterns in NFs and CAFs. To confirm fibroblast phenotype, expression of αSMA and collagen were determined in NFs and CAFs (Table 1). Next, we assessed endoglin expression by NFs and CAFs, which was not different between NFs and CAFs from the same patient, or between patients (Table 1). Although we have never observed endoglin expression on NFs in patients (supplementary Fig. S3), during *in vitro* culturing endoglin expression is highly upregulated. Since endoglin binds both TGF-β and BMP-9, expression of receptors involved in TGF-β/BMP signaling was determined (Table 1). No substantial differences were observed for receptor expression levels between the different patients. Additionally, when comparing NFs with CAFs from the same patient, only slight up- or downregulation of genes was detected. Certain CAF subpopulations can be distinguished based on distinct platelet derived growth factor receptor (PDGFR) subtype expression (*28*). Therefore we also analyzed gene expression of these receptors. However, these CAFs could not be divided into subgroups based on *in vitro* PDGFR expression (Table

1). As observed for the difference in endoglin expression *in vivo* and *in vitro*, other receptors could also be affected by culture conditions and therefore not represent *in vivo* expression profiles.

Table 1. Characterization of patient-derived NFs and CAFs

Gene	NF #1	CAF #1	NF #2	CAF #2	NF #3	CAF #3	NF #4	CAF #4
SMA	+++	+++	++	+++	+++	+++	+++	+++
Collagen-I	+++	+++	+++	+++	+++	+++	+++	+++
Endoglin	+++	++	+++	+++	+++	+++	+++	++
TGFβRII	++	++	++	++	++	++	++	++
BMPRII	++	++	++	+++	+++	++	++	++
ALK-1	++	+	+	++	++	++	++	++
ALK-2	++	++	++	++	++	++	++	++
ALK-3	++	++	++	++	+++	++	++	++
ALK-4	++	++	++	++	++	++	++	++
ALK-5	++	++	++	++	++	++	++	++
ACTRIIA	++	+	++	++	++	++	++	++
ACTRIIB	+	+	+	+	+	+	+	+
PDGFR-α	+++	+++	+++	+++	+++	+++	+++	+++
PDGFR-β	++	+	++	++	++	++	+	+

Expression of TGF-β and BMP receptors, PDGF receptors and characteristic fibroblast markers was determined in patient-derived NFs and CAFs. +, moderate expression (dCT 10-15); ++, high (dCT 5-10); +++, very high (dCT <5). All dCT values are expressed versus GAPDH. TGFβRII, TGF-β receptor type II; BMPRII, BMP receptor type II; ACTRIIA, activin receptor type IIA; ACTRIIB, activin receptor type IIB.

Endoglin expression is not regulated by ligand binding in fibroblasts

In endothelial cells, endoglin expression is at least partly regulated by TGF-β and BMP-9 ligand binding (*29, 30*). Upon binding, these ligands induce downstream signaling, which in the presence of endoglin results in phosphorylation of Smad1/5/8 and subsequent upregulation of endoglin expression. To assess endoglin signaling and subsequent regulation of its expression in fibroblasts, we stimulated CAFs with TGF-β or BMP-9 and determined downstream signaling and endoglin expression. Stimulation with BMP-9 resulted in fast and strong Smad1 phosphorylation, whereas TGF-β stimulation did not result in phosphorylation of Smad1 (Fig. 3A). Like for endothelial cells, BMP-9 stimulation induced expression of the Smad1 target gene inhibitor of differentiation-1 (ID-1). Canonical TGF-β signaling can occur through the TGF-β type I receptor ALK5, resulting in Smad2/3 phosphorylation, subsequently leading to induction of the Smad3 target gene plasminogen activator inhibitor-1 (PAI-1). Indeed, when CAFs were stimulated with TGF-β, PAI-1 expression was increased, whereas this was not affected upon BMP-9 stimulation (Fig. 3A). Expression of ID-1 was mildly increased by TGF-β, probably due to the subtle induction of Smad1 phosphorylation. Endoglin expression did not seem to be affected by ligand stimulation *in vitro*, both at the protein and mRNA level (Fig. 3A). However, endoglin expression *in vitro* is very high, which could hamper proper comparison to the *in vivo*

situation. Therefore, we also investigated a CAF subset with low endoglin expression.
In very low endoglin expressing CAFs, the effects of TGF-β stimulation on Smad1 phosphorylation and target gene expression were similar as observed for high endoglin expressing CAFs (Fig. 3B). BMP-9 stimulation, however, did not induce Smad1 phosphorylation or ID-1 gene expression (Fig. 3B), indicating that this pathway is endoglin-dependent. As was observed for the high endoglin expressing fibroblasts, endoglin expression itself was not affected by ligand stimulation (Fig. 3B).

Endoglin is indispensable for *in vitro* CAF survival and BMP-9 induced signaling

After establishing endoglin signaling in CAFs, its functional role was further evaluated. For this, different short hairpin RNA (shRNA) constructs targeting endoglin were introduced using lentiviral transduction. Knock down efficiency of constructs at RNA and protein level was confirmed in human endothelial cells (supplementary Fig. S4). All constructs reduced endoglin expression with at least 50% compared to a non-targeting control and did not affect endothelial cell morphology or survival. Transduced CAFs were passaged once after transduction with shRNA constructs and left to grow to confluency. CAFs transduced with either one of the shRNA constructs did not grow to confluency and, from 6 days after transduction, started to adopt a senescent morphology (Fig. 3C), eventually resulting in cell detachment and death. This phenotype was confirmed in two different CAFs and normal fibroblasts (data not shown), implying that endoglin is indispensable for CAF survival *in vitro*.

In order to still be able to study endoglin function in CAFs we took a different approach, using the endoglin neutralizing antibody TRC105. In endothelial cells, TRC105 binds specifically to the BMP-9 binding site in the orphan domain of endoglin, without affecting endoglin-dependent TGF-β signaling (*18*). Crosslinking experiments using radiolabeled TGF-β showed similar effects in CAFs; TGF-β binding to endoglin was not affected (data not shown). To investigate if BMP-9 binding is inhibited, and how downstream signaling was affected, CAFs were stimulated with TGF-β, BMP-9 and BMP-6 in the presence or absence of TRC105, and Smad phosphorylation was determined. Upon stimulation with TGF-β, both Smad1 and Smad2 phosphorylation were increased, either in the presence or absence of TRC105 (Fig. 3D). BMP-9 stimulation resulted in increased Smad1 phosphorylation, which was abrogated by TRC105. BMP-6 induces endoglin-independent Smad1 phosphorylation and was therefore not affected by TRC105. These results confirm that BMP-9-induced Smad1 phosphorylation is endoglin-dependent in CAFs and this can be inhibited by neutralizing endoglin with TRC105.

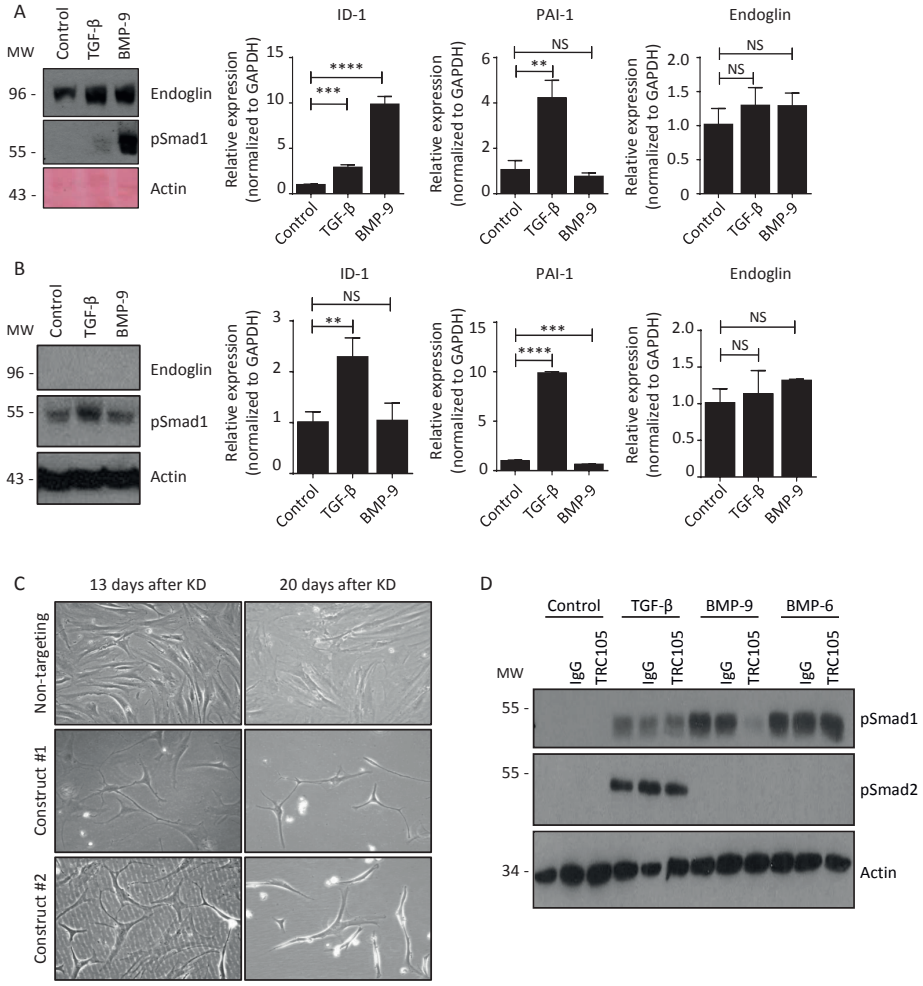


Figure 3. BMP-9 induced signaling in CAFs is endoglin-dependent. A. High endoglin expressing CAFs were serum starved overnight and subsequently stimulated with 5 ng/mL TGF-β or 0.1 ng/mL BMP-9 for 1 hour and downstream signaling and endoglin expression were assessed. BMP-9 stimulation increased Smad1 phosphorylation (pSmad1) and expression of its target gene ID-1. Stimulation with TGF-β slightly enhanced pSmad1 and ID-1 and highly increased expression of the Smad2 target gene PAI-1. Endoglin expression was unaffected by stimulation with either one of the ligands. B. Effects of TGF-β stimulation in low endoglin expressing CAFs were similar as observed for high expressing CAFs. BMP-9 stimulation did not show increased pSmad1 or ID-1. Ligand stimulation did not affect endoglin expression. C. Endoglin knock down using shRNA lead to senescent phenotype and eventual cell death. Validated using five different shRNA constructs and three CAFs. D. CRC CAFs were stimulated with TGF-β, BMP-9 or BMP-6 in the presence of TRC105 or an IgG control. TGF-β increased Smad2 and, to a lesser extent, Smad1 phosphorylation, independent of TRC105. BMP-9-induced pSmad1 was abrogated by TRC105. pSmad1 induced by BMP-6 was not affected by TRC105 treatment.

Endoglin enhances CAF invasion *in vitro*

Endoglin has been described to play an important role in endothelial cell migration, but also in non-endothelial cells several, sometimes contradictory, findings have been reported. To firstly establish the role of endoglin in cell migration, HEK293T cells, not endogenously expressing endoglin, were transfected with an endoglin expressing plasmid (Fig. 4A). Maximum invasion of HEK293T spheroids into a collagen-I matrix was assessed. The invasion pattern in HEK293T cells is different from fibroblast invasion, probably due to their epithelial origin. Figure 4B shows that, although at day 0 spheroids were of equal size, endoglin expressing HEK293T cells invaded the matrix significantly more after 24 hours than control cells. Spheroid diameter at day 1 was similar in both groups (data not shown), suggesting that invasion rather than proliferation is the main determinant of the observed effect. Based on these data, we further examined the role of endoglin in CAF migration. Since shRNA-mediated knockdown resulted in a lethal phenotype, we used high endoglin expressing CAFs (F2) and compared these with low endoglin expressing CAFs (F1, figure 4C). Endoglin mRNA expression levels differed 200-fold between the F1 and F2 CAFs, but was still detectable in F1 cells, also on the protein level, although very weak (data not shown and Fig. 4C). Spheroid forming ability was not dependent on endoglin expression levels. Spheroids were embedded in collagen-I matrix and left to invade for 48 hours. Figure 4D shows that F2 CAFs invaded the matrix to a higher extent than F1 CAFs, suggesting a role for endoglin in CAF invasion. To further confirm these findings, we overexpressed endoglin in low endoglin expressing F1-CAFs. Lentiviral transduction with an endoglin overexpression construct, increased expression more than 1000-fold compared with parental cells (Fig. 4E). Next, the invasive capacity was determined using transwell invasion assays on collagen-I coated inserts. After 24 hours, invaded cells at the lower side of the transwell insert were fixed, stained and the number of invaded cells was quantified. Figure 4F shows that endoglin overexpression significantly increased CAF invasion, when compared with the parental cells. These data show that endoglin expression is important for CAF invasion and that high endoglin expression increases the invasive capacity of CAFs.

To further study the role of endoglin in fibroblast migration we made used of murine embryonic fibroblasts isolated from endoglin *fl/fl* mice. Endoglin was deleted by transducing MEFs with a lentivirus expressing Cre recombinase. An empty vector virus was used as control. Transduced cells were selected using puromycin. Endoglin deletion did not appear to affect MEF proliferation rate and endoglin knock out MEFs remained viable for up to a maximum of three passages after transduction. Parental and endoglin knock out MEFs were characterized using RT-qPCR (supplementary Fig. S5), which confirmed strongly reduced endoglin expression (90% reduction, Fig. 5A). In transwell invasion assays, endoglin knock out resulted in significantly decreased MEF invasion through a collagen-I matrix, when compared with control MEFs (Fig. 5B). This observation supports the role of endoglin in fibroblast invasion *in vitro*.

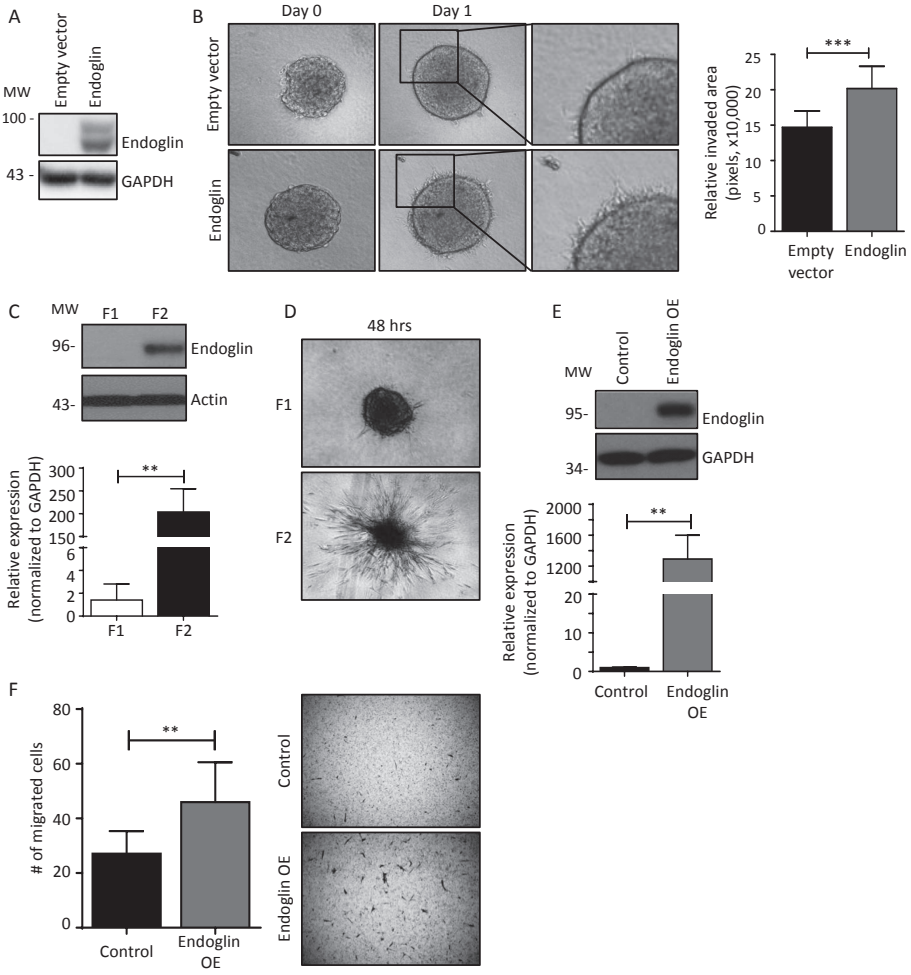


Figure 4. Endoglin enhances *in vitro* invasive capacity. HEK293T cells were transfected with an endoglin expressing construct (A) and left to form spheroids. In a 3D invasion assay, invasion of the collagen-I matrix after 24 hours was increased in cells expressing endoglin in the presence of 10% FCS (B). CAFs expressing high (F2) or very low (F1) levels of endoglin (C) were assessed for invasive properties. F2 CAFs invaded the collagen-I matrix more extensively than F1 CAFs in the presence of 10% FCS. Representative pictures of two experiments performed in triplicate (D). E. F1 CAFs were lentivirally transduced with an endoglin overexpression construct, increasing expression more than 1000-fold. Representative graph of three experiments. F. Invasive capacity was assessed during 24 hours by transwell migration through a collagen-I coated 8.0 µm membrane in the presence of 0.5% FCS. Endoglin overexpression significantly enhanced invasion, when compared with control F1 CAFs. Data represent mean of three independent experiments, performed in triplicate. **p≤0.01. ***p≤0.001.

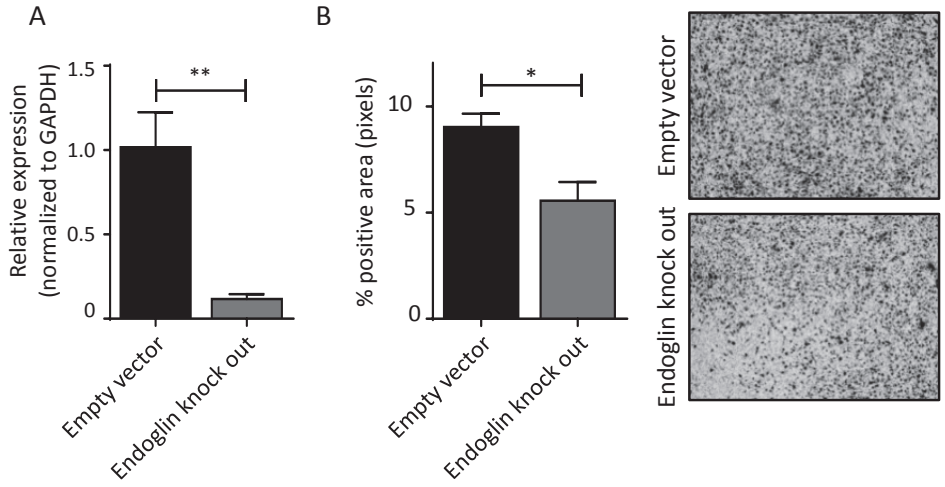


Figure 5. Endoglin knock out decreases invasion of embryonic fibroblasts. A. Lentiviral transduction of Cre recombinase expressing construct highly efficiently targeted endoglin expression in MEFs. Endoglin knock out MEFs proliferate at similar rates as empty vector control cells and remain viable for up to 3 passages after transduction. B. Endoglin knock out MEFs migrated significantly more in the presence of 0.5% FCS when compared with MEFs transduced with an empty vector in transwell invasion assays. Data represent mean of three independent experiments performed in triplicate. *p≤0.05, **p≤0.01.

Endoglin expression on fibroblasts promotes CRC liver invasion in zebrafish

To study the role of endoglin expression on fibroblasts in tumor metastasis in a more complex model, we developed a novel zebrafish model for CRC. In short, fluorescently labeled CRC cells were injected in the heart cavity of two-day old zebrafish embryos and ingrowth into the liver was followed over time. Since endoglin knock out was only feasible in MEFs, these cells were used as a model system with MC38 mouse CRC cells to study the interaction between fibroblasts and tumor cells without interspecies differences. MC38 cells are a metastatic cell line derived from stage-III adenocarcinomas in female C57BL/6 mice. Either in the presence or absence of MEFs, MC38 cells were injected and tumor growth and angiogenesis were monitored in time using confocal microscopy (Fig. 6A). Solid tumor-like structures were observed in both groups. From day 3 post injection, blood vessels are recruited towards the tumor. Co-injection of MC38 and MEFs resulted in decreased fish survival, compared with MC38 alone (Fig. 6B), which is probably caused by compromised liver function due to the ingrowth of tumor cells. Histological analysis revealed tumor cell invasion in the livers of both the MC38 alone and MC38+MEF co-injected groups (Fig. 6C). When the liver-infiltrating cells were closely analyzed, we found tumor cells (digitally clarified in blue) infiltrating in to the liver (digitally clarified in red) from day four after injection in the MC38 group and already two days after injection in the MC38+MEF co-injected group (Fig. 6C), suggesting enhanced tumor invasion in the presence of fibroblasts. Integration of MEFs in the tumors was detected by IHC using an

anti-αSMA antibody, which did not recognize zebrafish αSMA (Fig. 6D). Based on these results we propose that the presence of MEFs enhances the ingrowth of tumor cells into the liver, thereby compromising liver function, resulting in increased fish death.
To determine whether the observed effect in fish co-injected with MC38 and MEFs is due to endoglin expression on MEFs, experiments are ongoing using MC38 combined with endoglin knock out (ENG KO) MEFs.

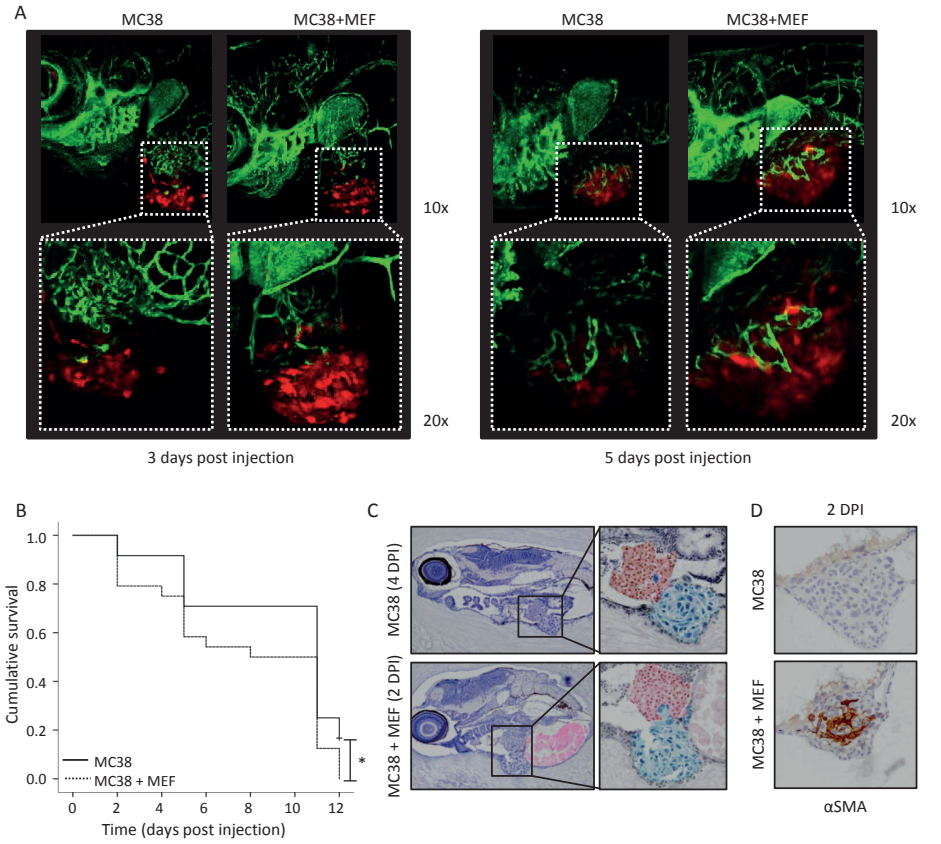


Figure 6. Invasion of the liver by colorectal tumor cells is regulated by fibroblasts. A. 2-day-old Tg(fli1:GFP) zebrafish embryos were injected with either 400 MC38 mouse CRC cells alone, or 200 MC38 cells in combination with 200 MEFs to induce tumor formation. Three and five days post injection tumors and tumor angiogenesis were visualized. B. Co-injection of MC38 cells and MEFs results in worse survival in zebrafish embryos (n=24/group). C. In MC38 alone injected fish, tumor infiltration into the liver was observed from day four on. Co-injection with MEFs accelerated this process and liver infiltration was seen from day two on. In order to clarify structures, digital staining of the liver (red) and tumor (blue) was applied. D. Immunohistochemical staining for αSMA showed integration of MEFs in MC38 tumors. *p≤0.05.

Endoglin targeting inhibits CRC liver metastasis in an *in vivo* mouse model

Finally, we assessed if neutralizing endoglin function can inhibit the metastatic spread of colorectal cancer cells. For this, we used a model for experimental metastasis formation using splenic injection of human CRC cells with and without CAFs in immunodeficient mice. Primary human CAFs were pretreated with TRC105 or an IgG control. Subsequently, mice were treated as shown in figure 7A. Metastatic spread was monitored by twice weekly bioluminescent imaging (Fig. 7B). Upon sacrificing the mice, livers were imaged *ex vivo* (Fig. 7C). In mice injected with tumor cells alone, treatment with TRC105 did not affect the number or extent of liver metastases, compared with IgG controls (data not shown), indicating that TRC105 does not affect metastasis formation by direct targeting of epithelial tumor cells. However, when tumor cells were co-injected with CAFs, TRC105 treatment showed a significant trend towards reduced liver metastases, compared with the IgG control group (Fig. 7D). These data suggest that targeting endoglin on CAFs results in less metastatic potential of the CRC cells. This might pose a novel target cell for TRC105 in addition to the endothelium and opens potential for targeting CRC metastatic spread.

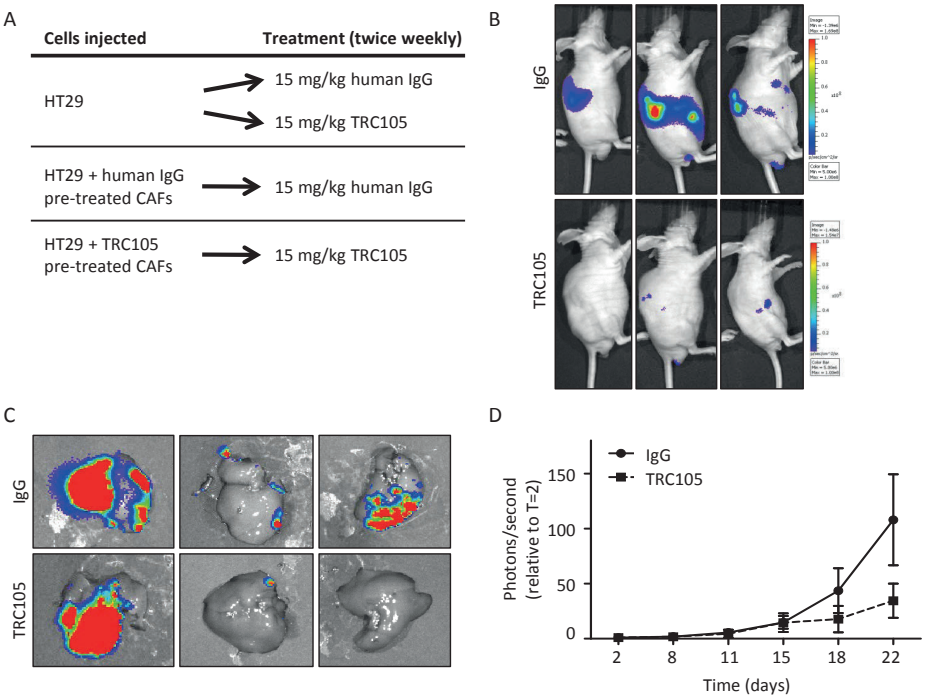


Figure 7. TRC105 treatment inhibits metastatic spread in a mouse model for CRC. A. Experimental set-up. Mice were injected with either HT29 alone, or in combination with CAFs, pre-treated with human IgG or TRC105. Two days after injection, treatment started as indicated for three weeks. B. Metastatic spread was followed in time using bioluminescent imaging. Mice co-injected with CAFs, treated with TRC105 presented with less metastases. C. *Ex-vivo* bioluminescence of livers at the end of the experiment. D. Quantification of bioluminescent signal over time showed a significant trend in decreased metastatic spread in TRC105 treated animals on day 22. Graph represents mean of 8 mice/group.

Discussion

In this study we show that CAF-specific endoglin expression is predictive for the development of metastatic disease in early stage CRC and is detected in metastatic lesions of CRC patients with advanced metastatic disease. Additionally, we revealed the role of endoglin in CAF invasion *in vitro* and in a novel zebrafish model for CRC. In an *in vivo* CRC metastasis model, treatment with the endoglin neutralizing antibody TRC105 resulted in decreased metastatic spread of CRC cells, when co-injected with CAFs.

Endoglin is of crucial importance for development of the vasculature as underlined by embryonic lethality of endoglin knockout mice. In addition, our study shows the importance of endoglin expression in fibroblast survival. *In vitro* endoglin knock down induced cell death in primary fibroblasts. Romero and colleagues reported the inability to culture primary prostate CAFs, isolated from endoglin heterozygous mice, whereas prostate cancer-derived CAFs from endoglin wild type mice could easily be propagated *in vitro* (*31*). We observed that, although normal fibroblasts *in vivo* do not express endoglin, endoglin expression is highly upregulated upon *in vitro* culturing. Lower expression of endoglin by genetic deletion or shRNA-mediated knock down, might prevent high level endoglin expression needed for *in vitro* survival. This differential expression of endoglin on fibroblasts *in vitro* and *in vivo* could hamper accurate molecular studies on endoglin expression in fibroblasts and should be kept in mind when assessing *in vitro* results.

In our *in vitro* assays, endoglin expression regulated CAF invasion in collagen-I matrix. In endothelial cells, endoglin has been shown to be important for proliferation (*32*) and migration (*33*), subsequently leading to an angiogenic response. Deletion of endoglin resulted in decreased Smad1 phosphorylation and TGF-β-induced endothelial cell migration was enhanced (*34*). The latter effect can probably be ascribed to the inhibitory effect of endoglin on TGF-β/ALK5 signaling (*33*), which is alleviated upon endoglin knock out. We have recently observed increased Smad2 phosphorylation in endothelial cells when neutralizing endoglin with TRC105, which resulted in decreased angiogenic capacity *in vitro* and *in vivo* (*25*). Moreover, Tian et al. showed that endoglin on endothelial cells plays an important role in mediating interactions between the ECM component fibronectin and its cellular receptor integrin α5β1, crucial for adhesion and migration (*35*). In fibroblasts, interactions between fibronectin and integrin α5β1 also play an important role in cellular migration (*36*), although the importance of endoglin in this interaction was not assessed in these cells.

The stimulatory effect of endoglin on CAF invasion *in vitro* and its localization at the invasive border of colorectal tumors, suggests a role for endoglin-expressing CAFs in tumor metastasis. When we assessed lymph node and liver metastases from CRC patients, we indeed observed the presence of endoglin positive CAFs in these lesions. Although contradictory research has been published, co-migration of CAFs with CRC cells to the liver has been reported. In an experimental model for CRC metastasis, GFP expressing CAFs were localized in liver metastases and enhanced the formation of these metastatic lesions (*37*). In

prostate cancer, it was observed that although endoglin heterozygosity increased primary tumor growth, the number of metastases was lower than observed in wild type mice (*31*). More recently, Anderberg et al. reported increased metastatic spread of pancreatic tumors in endoglin heterozygous mice. Additionally, endothelial cell-specific endoglin knock out also resulted in increased metastatic spread in a subcutaneous model for lung cancer (*38*). This might seem contradictory to our findings, but these studies describe effects on metastatic spread in mouse models with comprised endothelial integrity due to endoglin heterozygosity, whereas our experiments specifically assessed the role of endoglin on CAFs. Moreover, TRC105 does not affect endothelial integrity (unpublished data). Endoglin deficiency *in vivo* results in a phenotype resembling hereditary hemorrhagic telangiectasia, which includes increased vascular permeability (*39, 40*), possibly facilitating tumor cell intra- and extravasation and therefore metastasis.

Interestingly, endoglin heterozygous prostate tumors presented as being non-fibrotic, indicating a lack of CAFs, as confirmed by absence of αSMA expressing cells (*31*). Moreover, we have recently reported in a mouse model for breast cancer that treatment with TRC105 reduced the number of metastasis, which was accompanied by a decrease in the αSMA-positive stromal component (*25*). These observations underline the relevance of CAFs in metastatic spread. When we consider tumors to be wounds that do not heal (*41*), the fibrotic response is very important and results in increased matrix deposition, fibroblast proliferation and angiogenesis, all contributing to tumor progression and metastasis. The role of endoglin on fibroblasts in cardiac fibrosis was described by Kapur and colleagues, who showed that endoglin was upregulated during cardiac fibrosis and reduced endoglin expression prevented cardiac fibrosis *in vivo*. Moreover, overexpression of soluble endoglin, acting as a ligand trap, showed similar effects (*42*), suggesting an important role for endoglin in fibrotic response. However, these experiments were performed in endoglin heterozygous mice and can therefore not exclude an additional role of other endoglin-expressing cell types in this process, next to fibroblasts.

In patients, we report a relation between high endoglin expression on CAFs at the invasive tumor borders and decreased metastasis-free survival in stage-II CRC, implying the involvement of this CAF subset in tumor invasion and metastasis. Although the overall score of endoglin expression on CAFs in the stage-III cohort was higher than for stage-II, we did not find a correlation with disease-free or overall survival. Since it appears that endoglin on CAFs is a predictive factor for development of metastasis and stage-III patients already present with local metastases, the higher endoglin expression and lack of correlation is not surprising. Moreover, the presence of αSMA-expressing fibroblasts was reported to correlate with lymphatic invasion in early stage CRC (*43*). Here, we show co-localization of endoglin and αSMA expression, suggesting an overlapping cell population. Already presenting with metastasis, stage-III patients have yet passed this stage, posing another possible explanation for our observations.

In summary, the data presented here point to a crucial involvement of endoglin expressing CAFs in CRC invasion and metastasis and could therefore prove to be a potential therapeutic target. Additionally, CAF-specific endoglin expression could be a potential prognostic factor in early stage CRC. In a phase I study, TRC105 showed clinical efficacy on pre-existing metastases in two patients (*17*). Combined with our recently published data that adjuvant TRC105 treatment decreased metastatic spread in an *in vivo* breast cancer model (*25*), and our observations that endoglin is involved in CAF invasion, targeting endoglin on CAFs, in addition to the endothelium, could be a potent approach in preventing metastasis formation.

Acknowledgements

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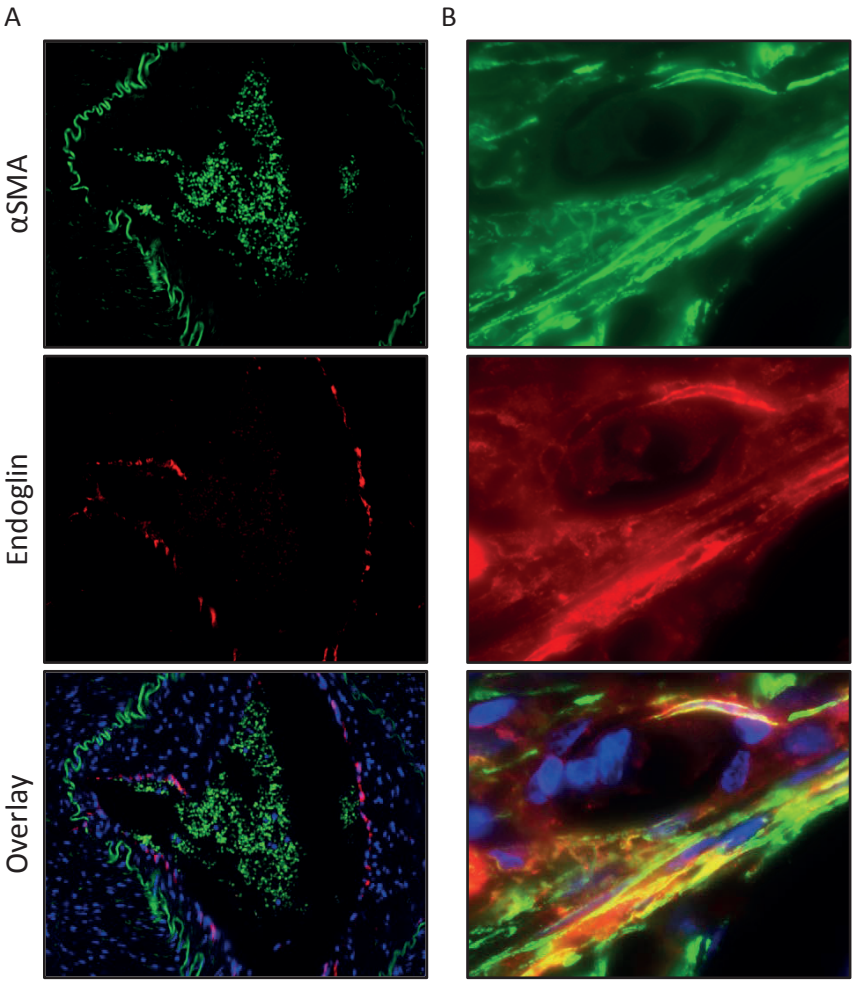
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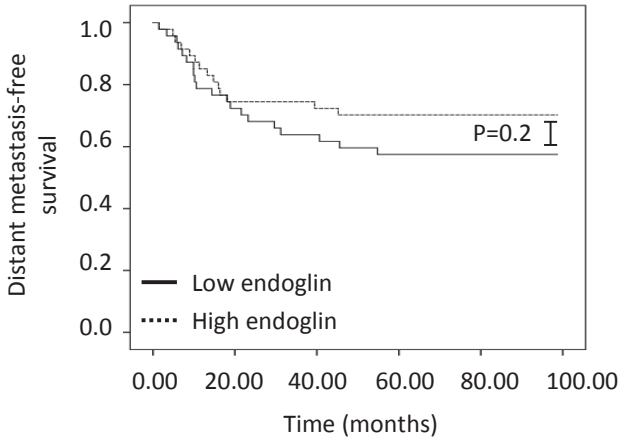
Supplementary data

Supplementary Table S1 qPCR primer sequences.

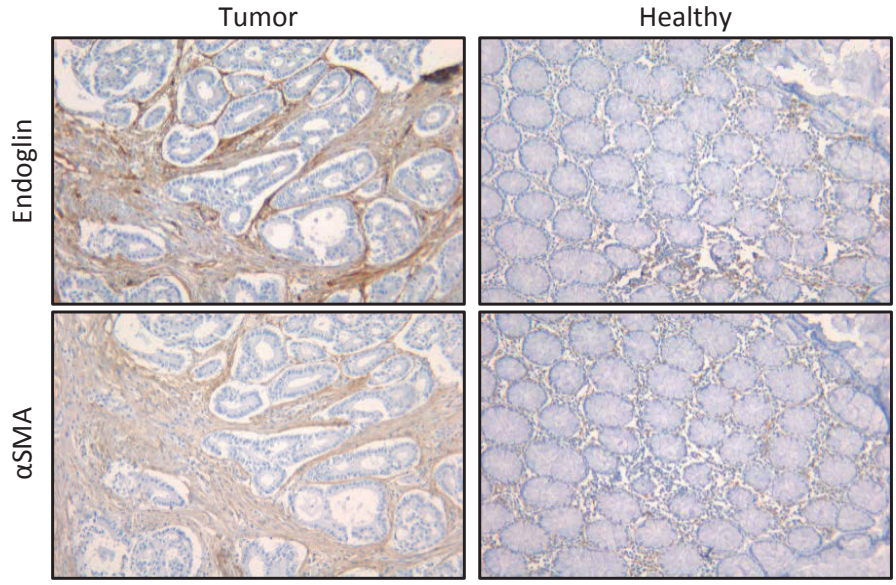
Gene	Forward	Reverse
Human *GAPDH*	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Human *endoglin*	CCGAGAGGTGCTTCTGGTCC	GTGCAGTGGGATTCCCAGG
Human *TGF-β type II receptor*	TTCAGAAGTCGGATGTGGAAATG	GTTGTAAGTGACTATCATGTCGT
Human *BMP type II receptor*	AACTGTTGGAGCTGATTGGC	CGGTTTGCAAAGGAAAACAC
Human *ALK-1*	ATGACCTCCCGCAACTCGAT	TAGAGGGAGCCGTGCTCGT
Human *ALK-2*	TGCCTTCGAATAGTGCTGTC	CATCAAGCTGATTGGTGCTC
Human *ALK-3*	GGGGTCCGGACTTATGAAA	TACGACTCCTCCAAGATGTGG
Human *ALK-4*	GCTCGAAGATGCAATTCTGG	TTGGCATACCAACACTCTCG
Human *ALK-5*	ACGGCGTTACAGTGTTTCTG	GCACATACAAACGGCCTATCT
Human *activin type IIA receptor*	GCATCACAAGATGGCCTACC	CCAGGCAAACTGTAGACTTC
Human *activin type IIB receptor*	ATGTGGACATCCATGAGGAC	TGAAGATCTCCCGTTCACTC
Human *PDGF receptor β*	TGCCTCCGACGAGATCTATG	GGGTGGTCACTCCTCAGAAA
Human *PDGF receptor α*	CTGGACACTGGGAGATTCGG	CCGCAATGAATGTCCCACAC
Human αSMA	CCGGGAGAAAATGACTCAAA	GAAGGAATAGCCACGCTCAG
Human *collagen-I*	CAGCGCCTTCACCTACAGC	TTTGTATTCAATCACTGTCTTGCC
Human *PAI-1*	GCCAACAAGAGCCAATCACA	AGGCAAGCAAGGGCTGAAG
Human *ID-1*	CTGCTCTACGACATGAACGG	GAAGGTCCCTGATGTAGTCGAT
Mouse *GAPDH*	ATCACTGCCACCCAGAAGAC	ATGAGGTCCACCACCCTGTT
Mouse *endoglin*	TGAATGGCAACCACGAGC	GCAGGATGAGAATGGCGTC
Mouse *TGF-β type II receptor*	GGGCATCGCTCATCTCCAC	TCCCTGTGAACAATGGGCAT
Mouse *BMP receptor II*	CCACAACCCAGTATGCCAATG	TGGGACCTATGTGCCACTATGTT
Mouse *ALK-3*	TACCAGCAGGTGTTAATGCG	TGGCACATTTCAGGAAGTCA
Mouse *ALK-5*	GCCATAACCGCACTGTC	ATGGGCAATAGCTGGTTTT
Mouse αSMA	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA



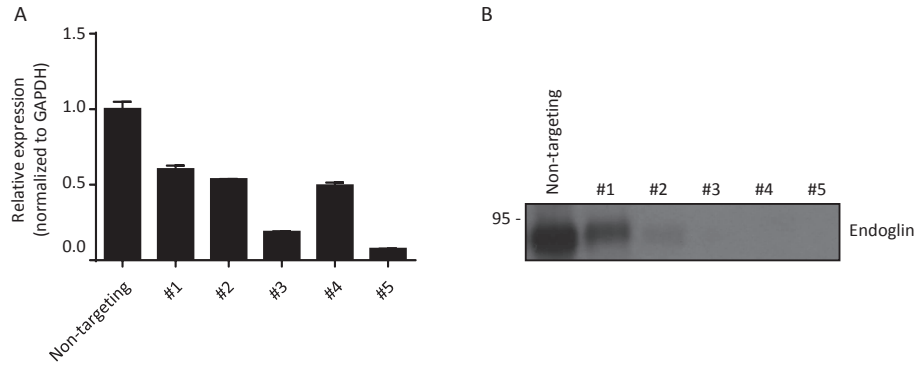
Supplementary figure S1. A. αSMA expression (green) on smooth muscle cell layer surrounding a large vessel. Endoglin (red) is specifically expressed by angiogenic endothelial cells within the blood vessel. B. High magnification of endoglin expression on CAFs.



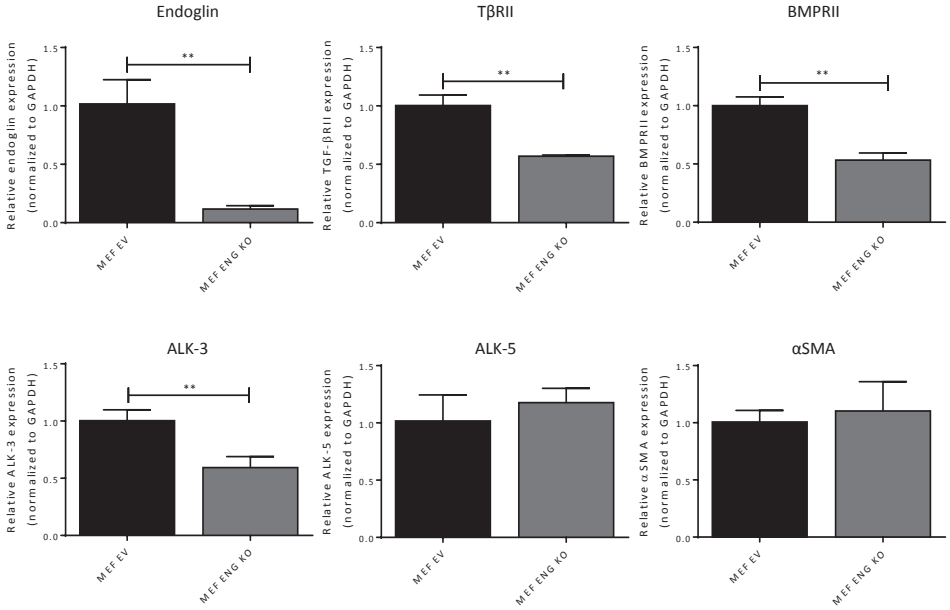
Supplementary figure S2. Endoglin expression on CAFs at the invasive border does not correlate with survival in stage-III CRC. When endoglin expression on CAFs at the invasive borders was related to distant metastasis-free survival, no correlation between the two variables was found.



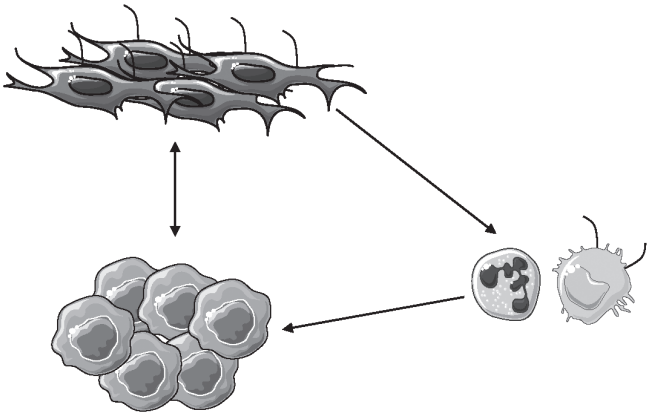
Supplementary figure S3. Fibroblasts in normal colorectal tissue do not express endoglin. Normal and tumor tissues from the same patient were stained for endoglin and αSMA expression to assess endoglin expression on (cancer-associated) fibroblasts. αSMA-positive fibroblasts in normal colonic tissue did not show endoglin expression. Pictures are representative for all patient tissues discussed in this manuscript. Tissue from patient #4, described in table 1.



Supplementary figure S4. Endoglin knock down in human endothelial cells. Short hairpin RNA constructs were introduced in human endothelial cells by lentiviral transduction. All constructs reduced endoglin expression at mRNA level with at least 50% (A), which was reflected at the protein level (B).



Supplementary figure S5. Characterization of TGF-β and BMP receptors in MEFs. After lentiviral transduction with a Cre recombinase expressing construct (ENG KO) or empty vector control (EV), cells were selected with puromycin. Effects of endoglin knock out on other receptors in TGF-β/BMP signaling were determined. Expression of endoglin, TGF-β receptor II (TβRII), BMP receptor II (BMPRII) and TGF-β type I receptor ALK-3 were significantly decreased upon Cre recombinase transduction. TGF-β type I receptor ALK-5 expression was not affected. αSMA levels did not change after Cre recombinase transduction.



Chapter 8

Fibroblast-specific endoglin knock out increases formation of intestinal pre-malignant lesions

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Manuscript in preparation

Abstract

Disruption of the intricate balance in paracrine communication between epithelial cells and intestinal stromal cells disturbs intestinal homeostasis and can result in spontaneous polyp formation. As during homeostasis, interactions between epithelial tumor cells and the tumor stroma play an important often pro-tumorigenic role during colorectal cancer (CRC) progression. Previously, we showed the importance of the transforming growth factor-β (TGF-β) co-receptor endoglin on cancer-associated fibroblasts (CAFs) in CRC metastasis, suggesting a role in the interaction between tumor cells and CAFs. To determine the role of endoglin on fibroblasts in intestinal tumorigenesis, we generated a tamoxifen-inducible, fibroblast-specific endoglin knock out mouse. Tumor formation was induced using azoxymethane followed by dextran sodium sulphate (AOM/DSS). We observed a high increase in the number of AOM/DSS-induced lesions upon fibroblast-specific endoglin deletion. Lesion size was not affected by endoglin deletion. In lesions from endoglin knockout mice, both total stroma content and the number of activated fibroblasts were increased, although this did not affect total collagen deposition. Additionally, increased infiltration of macrophages and neutrophils in tumors was observed. Moreover, expression profiles of immunoregulatory genes were changed in endoglin knock out fibroblasts *in vitro* and in lesions from mice after fibroblast-specific endoglin deletion. Together, these data suggest an important role for endoglin on fibroblasts during tumorigenesis, possibly via altered recruitment of macrophages and neutrophils.

Introduction

The intestinal epithelium is very dynamic, with a high proliferation rate in the colonic crypts and differentiation and apoptosis at the luminal surface of the intestine (*1, 2*). This homeostatic process is tightly regulated by paracrine signaling between epithelial and stromal cells in the intestine. Major players in intestinal homeostasis are the bone morphogenetic protein/transforming growth factor-β (BMP/TGF-β) and Wnt signaling pathways (*3-6*).

As for intestinal homeostasis, paracrine interactions between malignant epithelial cells and their microenvironment play a crucial role in colorectal cancer (CRC) progression and metastatic spread (*7, 8*). The tumor microenvironment (TME), consists of endothelial cells, immune cells and cancer-associated fibroblasts (CAFs) (*9*). CAFs are a main component of the TME and have been shown to be involved in tumor progression and invasiveness, e.g. by remodeling the extracellular matrix (ECM), secreting proteases and regulating the immune status of the tumor (*10-12*). CAFs are a heterogeneous cell population with a fibroblast morphology, expressing vimentin, α-Smooth Muscle Actin (αSMA) and fibroblast activation protein (FAP) (*13*). Although extensively studied, the cell-of-origin of CAFs is still under debate. One commonly accepted hypothesis is that local fibroblasts are activated under the influence of tumor-derived cytokines, mainly TGF-β (*14-16*).

The TGF-β co-receptor endoglin is highly expressed on activated endothelium and was shown to be indispensable for developmental angiogenesis (*17-19*). In endothelial cells, endoglin is involved in signal transduction of its ligands TGF-β and BMP-9 (*20, 21*), ultimately leading to a pro-angiogenic phenotype, characterized by increased endothelial proliferation and migration (*22-25*).

We and other groups have observed that, next to endothelial cells, endoglin is expressed on CAFs, mainly at the invasive borders of solid tumors (chapter 7). Endoglin on CAFs can play an important role in CRC metastasis, which is further strengthened by the fact that treatment with the endoglin neutralizing antibody TRC105 decreased metastatic spread in an *in vivo* model for CRC metastasis, rendering endoglin on CAFs a potential therapeutic target in CRC (chapter 7).

In this project we addressed the role of endoglin on fibroblasts during CRC initiation and progression, using the azoxymethane/dextran sodium sulphate (AOM/DSS) model (*26*). This most commonly used model for colitis-associated cancer generates tumors which show resemblance to human CRC (*27*).

We generated a tamoxifen-inducible collagen1α1-specific endoglin knock out mouse ($ENG^{Fib-/-}$). When we chemically induced tumor formation, a significant increase in the number of lesions was observed in $ENG^{Fib-/-}$ mice, when compared with control mice. Tissue analysis and *in vitro* experiments revealed that collagen1α1-specific, further referred to as fibroblast-specific, endoglin knock out resulted mainly in an expansion of the stromal compartment of lesions. Moreover, deletion of endoglin in fibroblasts resulted in enhanced recruitment of macrophages and neutrophils to AOM/DSS-induced lesions. Together, these

data imply that endoglin expression on fibroblasts might play a role in initiation of CRC, potentially by regulating immune cell infiltrate in early lesions.

Materials and methods

Cell culture, preparation of CM and signaling assays

Mouse fibroblasts and the mouse CRC cell line MC38 (*28*) were cultured in in DMEM/F12, supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 50 µg/mL gentamycin, 100 IU/mL penicillin and 100 µg/mL streptomycin (all ThermoFisher, Waltham, MA, USA). Mouse CRC cell line CT26 (*29*) was maintained in RPMI 1640, supplemented with 10% FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin (all ThermoFisher). Murine embryonic fibroblasts (MEFs) were obtained from E12.5 embryos as described before (*30*), from an endoglin flox/flox mouse strain in which exons 5 and 6 are flanked by LoxP sites (*31*). MEFs and the mouse myoblast cell line C2C12 were maintained in DMEM, supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin and 100 µg/mL streptomycin (all ThermoScientific). Constructs expressing Cre recombinase (pLV.mPGK.iCRE.IRES.PuroR, kindly provided by Dr. M. Goncalves, Dept. Molecular Cell Biology, Leiden University Medical Center) or an empty vector control were delivered using lentiviral transduction using polybrene (4 µg/mL, Hexadimethrine bromide, Sigma Aldrich) to 80% confluent MEFs and after 48 hours, transduced cells were selected by 1.5 µg/mL puromycin (Sigma Aldrich).

Conditioned medium (CM) from MEFs was prepared by serum starving subconfluent cells for four days. CM used for proliferation assays was two-fold diluted with culture medium, containing 5% FCS.

BMP signaling was determined using a Bre-Luc reporter construct as described earlier (*32*) and TGF-β signaling was assessed as described before (*8*). In short, MC38 or C2C12 cells were transfected using polyethylenimine (PEI; Polysciences Inc., Warrington, PA, USA). After four hours, medium was changed to normal culture medium and the next day cells were serum-starved overnight. Cells were stimulated with 5 ng/mL TGF-β (*33*), 100 ng/mL BMP-6 (PeproTech, London, UK), or conditioned medium from MEFs. After 6 hours of stimulation, cells were washed, lysed and luciferase activity was measured according to manufacturers' instructions (Promega, Leiden, The Netherlands).

MTS proliferation assay

5000 CT26 or MC38 cells were seeded in 96-well plates in triplicate. After 16 hours, medium was replaced with 100 µL CM, from either control or endoglin knock out MEFs or with non-conditioned medium. At indicated time points 20 µL MTS substrate (Promega, Madison, WI, USA) was added to each well and absorbance was measured at 490 nm using a VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA).

Mice

All animal experiments were approved by the Dutch animal ethics committee. Next to its expression in osteoblasts (*34*), collagen1α1 is expressed in fibroblasts (*35-37*), including those of the intestine (*38*). In this study we have used the collagen1α1 promoter in order to delete endoglin from fibroblasts, without affecting its expression on endothelial cells. Collagen1α1-CreERT2 mice were purchased from Jackson Laboratory (strain B6.Cg-Tg(Col1a1-cre/ERT2)1Crm, Bar Harbor, ME, USA). ENG*fl/fl* mice in which exons 5 and 6 of the endoglin gene are flanked by LoxP sites were generated by Allinson et al. (*31*). Before tamoxifen induction mice were divided into two groups, based on sex and body weight. Cre-mediated recombination was induced at 8 weeks of age by oral administration of 50 uL tamoxifen (100 μg/mL, Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in sunflower oil, on three consecutive days. Control mice had the same genotype, but were not treated with tamoxifen. Collagen1α1-CreERT2.ENG*fl/fl* and ENG*fl/fl* mice were used for control experiments as described. Mice were genotyped by PCR for the presence of the Cre recombinase and endoglin LoxP gene using the following primers; Cre recombinase Fw 5'-ACGAGTGATGAGGTTCGCAA -3', Cre recombinase Rev 5'-AGCGTTTTCGTTCTGCCAAT -3', endoglin LoxP Fw 5'-CCACGCCTTTGTCCTTGC-3', endoglin LoxP Rev 5'-GACGCCATTCTCATCCTGC-3' (all Invitrogen Carlsbad, CA, USA).

AOM/DSS model

Two weeks after tamoxifen induction male and female Collagen1α1-CreERT2.ENG*fl/fl* mice received one intraperitoneal injection with 10 mg/mL azoxymethane (AOM; Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in saline. Two days later, the first 7-day cycle with 1.5% dextran sodium sulphate (DSS; MP Biomedicals, Santa Ana, CA, USA) dissolved in drinking water, supplemented with artificial sweetener (Natrena), was started. After seven days, drinking water was changed to standard conditions for 14 days. This three-week cycle was repeated twice more during the experiment. During DSS cycles mice weight and overall health were monitored daily, while during the "off" period animals were weighted and checked every other day. Two weeks after the third DSS cycle, mice were sacrificed and blood and tissue samples were collected. Colons were fixed in 4% formaldehyde and photographed. Lesion volume was measured in photographed colons by ImageJ (National Institute of Health) and tumor volume was calculated (tumor volume = (width^2 × length)/2).

RT-qPCR

Tissue samples were disintegrated using a TissueLyser (Qiagen, Hilden, Germany) and RNA was isolated using Nucleospin RNA kit (Bioké, Leiden, The Netherlands), according to manufacturers' instructions. For *in vitro* experiments, MEFs were grown to confluency, harvested and RNA was isolated as described above. RNA concentration and purity were determined using NanoDrop 3300 (Thermo Scientific, Breda, The Netherlands). Complementary DNA synthesis was performed using 1 μg RNA using RevertAid First Strand cDNA synthesis kit, according to manufacturers' instructions (ThermoScientific).

Quantitative PCR analyses were performed as described before (*8*), using primers as described in supplementary table S1 (Invitrogen). All values were normalized by *GAPDH* expression.

Fluorescence-activated cell sorting

Tumor material was minced with scalpels and digested with Liberase (Roche, Basel, Switzerland) according to manufacturer's protocol. Single cell suspensions were made and stained with antibodies against CD45, CD11b (both eBioscience, Vienna, Austria), F4/80, Ly6C and Ly6G (all BioLegend, San Diego, CA, USA). FACS analysis was performed on a LSR II system (Becton Dickinson, Breda, The Netherlands). Data was analyzed using FlowJo data analysis software (FlowJo, Ashland, OR, USA).

Tissue analysis

Immunohistochemical stainings were performed as described previously (*39*), using primary antibodies against vimentin, cleaved caspase 3 (both Cell Signaling Technologies, Danvers, MA, USA), αSMA (Progen, Heidelberg, Germany), Ki67 (Millipore, Amsterdam, The Netherlands), Ly6G (BioLegend), F4/80 (eBioscience) and endoglin (R&D systems, Abington, UK). For quantification of total collagen, tumor sections were stained with Sirius red (Klinipath, Duiven, The Netherlands). In short, paraffin sections were deparaffinized, stained with 0.1% Sirius red in picric acid, washed in 0.01M HCl and subsequently dehydrated and mounted in entellan. Three to five representative pictures per mouse were taken with an Olympus BX51TF microscope (Olympus Life Science Solutions, Zoeterwoude, The Netherlands) and staining was quantified using ImageJ software (National Institutes of Health). Quantification of macrophage infiltration was scored based on F4/80 staining. Score 1; ≤5% stroma positive, score 2; 5-25% stroma positive, score 3; 25-50% stroma positive, score 4; ≥50% stroma positive.

Statistical analysis

Tissue analysis was performed in a blinded manner by two independent observers. Data indicate mean ± s.e.m. or SD, as indicated in figure legends. Differences between groups were calculated using Students' *t*-test or Mann-Whitney analysis. P-values ≤0.05 were considered statistical significant.

Results

Fibroblast-specific endoglin knock out enhances AOM/DSS-induced adenoma formation

In order to assess the effect of inducible collagen1α1-specific endoglin deletion on AOM/DSS-induced tumor formation, collagen1α1-Cre-ERT2 mice were crossbred with ENG*fl/fl* mice, generating collagen1α1-CreERT2.ENG*fl/fl* mice. Cre-mediated recombination

was induced in 8-week old animals by oral administration of tamoxifen, generating ENG$^{Fib-/-}$ mice. Animals were exposed to the AOM/DSS protocol as described in figure 1A. Throughout the course of the experiment, mice in both groups showed weight loss during DSS supplementation, which recovered during the "off" periods (Supplementary fig. S1A). Upon termination of the experiment, the number of lesions in the colorectum was quantified. The occurrence of colonic lesions was strongly increased in the ENG$^{Fib-/-}$ group when compared with control mice (Fig. 1B and C). Although the number of tumors differed, average lesion size was similar in both groups (Fig. 1D). In the ENG$^{Fib-/-}$ group serious complications, including rectal blood loss, rectal prolapse or substantial weight loss, were more often observed, reflecting the more severe phenotype resulting in premature animal sacrifice. The number of lesions per mouse was not dependent on sex of the animals (Supplementary fig. S1B). Lesions were analyzed by H&E staining and characterized by a pathologist as adenomas with high grade dysplasia (Fig. 1E). Throughout the course of our experiments, no neoplastic growth at other locations than the colorectum was observed. These data suggest that endoglin on fibroblasts plays an important role in chemically-induced colorectal tumorigenesis.

Fibroblast-specific endoglin knock out does not induce spontaneous adenoma formation

To exclude that endoglin deletion in fibroblasts results in spontaneous neoplastic growth, collagen1α1-CreERT2.ENG*fl/fl* mice were induced with tamoxifen. Animals were housed for 13 weeks, identical to the time course of the experiment, and were not treated with AOM or DSS. At the end of the experiment lesion formation in the colorectum was assessed. In both ENG$^{Fib-/-}$ and control mice, no lesions in the colorectum were observed (Supplementary fig. S1C). Additional histological analysis did not reveal any morphological changes in the colon (data not shown). This suggests that fibroblast-specific endoglin knock out does not result in spontaneous neoplastic growth during the course of our experiments.

Additionally, to assess the effect of tamoxifen administration on tumor induction, ENG*fl/fl* mice without the Cre recombinase, as a negative control, received oral tamoxifen and were subsequently exposed to the AOM/DSS regimen as described in figure 1A. After 13 weeks, the number of lesions in the colorectum of tamoxifen-induced ENG*fl/fl* mice was similar to non-induced Collagen1α1-CreERT2.ENG*fl/fl* mice treated with AOM and DSS (Supplementary fig. S1C). This implies that tamoxifen administration does not affect AOM/DSS-induced lesion formation in our model.

Fibroblast-specific endoglin knock out increases stromal content

Disturbance of paracrine signaling between stromal and epithelial cells can result in increased proliferation or decreased apoptosis rates, thereby contributing to tumor formation. Therefore, we analyzed adenomas from control and ENG$^{Fib-/-}$ mice to assess changes in these processes. First, we determined the proliferation rate in AOM/DSS-induced lesions using Ki67 expression, the cellular marker for proliferation, by immunohistochemistry

and quantified the number of Ki67 positive cells. Upon quantification, similar numbers of proliferating cells were observed in both groups (Fig. 2A). Next, we stained for the apoptotic marker cleaved caspase 3 and counted the number of apoptotic cells in the adenomas. The number of apoptotic cells was similar in both control and ENG$^{Fib-/-}$ mice (Fig. 2B).

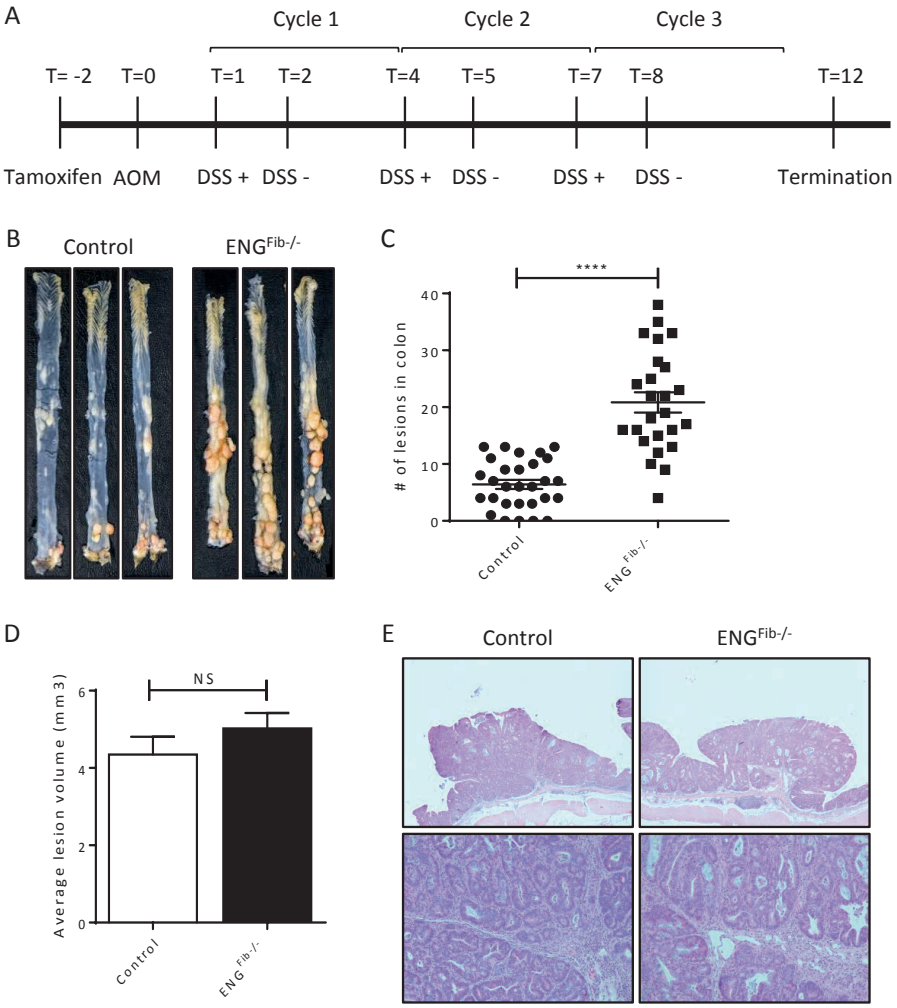


Figure 1. Fibroblast-specific endoglin knock out enhances AOM/DSS-induced neoplastic growth. A. Experimental set-up. At 8 weeks of age, mice were induced with tamoxifen. After two weeks, AOM was injected, followed by three 21-day DSS cycles. Two weeks after the last DSS cycle, experiments were terminated. B. Representative pictures of colons obtained from control and fibroblast-specific endoglin knock out (ENG$^{Fib-/-}$) mice at end of the experiment. C. Neoplastic growth was highly increased in ENG$^{Fib-/-}$ mice, although size of the lesions did not differ between the groups (D). E. Histological analysis of AOM/DSS-induced lesions revealed adenomas with high grade dysplasia. Graphs represent mean of 29-25 mice/group from two independent experiments. ****P≤0.0001.

Since fibroblasts are the major component of the tumor stroma, the effect of fibroblast-specific endoglin deletion on total stroma content of the lesions was assessed by vimentin staining. Lesions from $ENG^{Fib-/-}$ mice showed a significant increase in vimentin positive cells, when compared with control mice (Fig. 2C). Next, αSMA staining was used to determine the proportion of activated fibroblasts in the stroma. As observed for vimentin, the percentage of αSMA-positive content was increased in $ENG^{Fib-/-}$ lesions compared with the control (Fig. 2D). Although more αSMA-positive fibroblasts were observed in $ENG^{Fib-/-}$ lesions, total collagen production as determined using Sirius red staining, did not differ between the two groups (Fig. 2E). Together, these data imply that, without affecting the balance between proliferation and apoptosis in intestinal epithelium, expansion of the stromal compartment might contribute to neoplastic growth in our model.

Enhanced immune infiltration in fibroblast-specific endoglin knock out adenomas

Cancer-associated fibroblasts have been shown to secrete chemoattractants and hereby regulate recruitment of immune cells to the tumor (*12*). Moreover, immune cells are proven to be an important part of the tumor microenvironment and promote tumor initiation and progression (*40-43*). Therefore, we determined the extent and composition of immune infiltrate in the AOM/DSS-induced lesions. Upon termination of the experiment, neoplastic tissues of three mice per group were subjected to flow cytometry. Total immune infiltrate, based on CD45 expression, was not significantly changed between the control and $ENG^{Fib-/-}$ group (Fig.3A). However, when we determined the percentage CD11b-expressing cells (monocytes and macrophages) in the CD45+ population, increased infiltration was observed in $ENG^{Fib-/-}$ lesions (Fig.3B). In order to further specify this CD11b+ population, the abundance of macrophages was determined using Ly6C expression. Although Ly6C+ monocytes appeared to be increased in the $ENG^{Fib-/-}$ group, this did not reach statistical significance (Fig.3C). The percentage of Ly6C- macrophages, though, was significantly increased in $ENG^{Fib-/-}$ lesions, when compared with controls (Fig.3D). Increased macrophage recruitment to $ENG^{Fib-/-}$ lesions was confirmed by quantification of immunohistochemical staining for the macrophage marker F4/80 (Fig.3E).

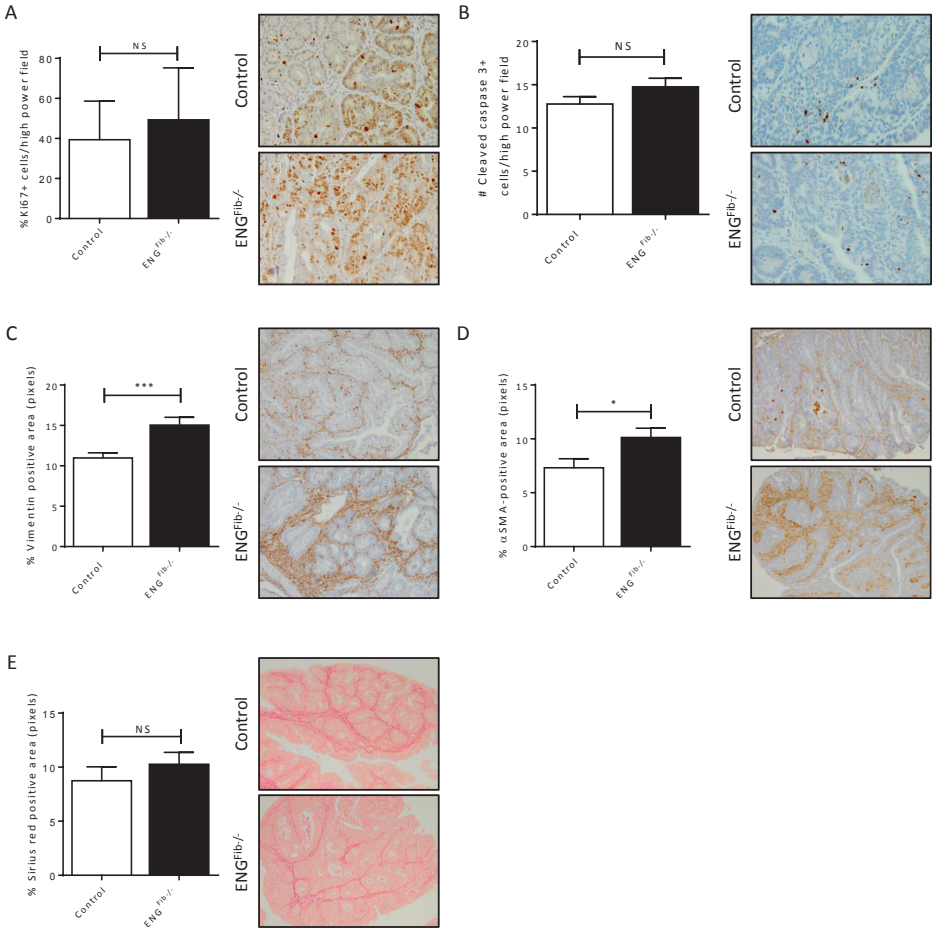


Figure 2. Endoglin deletion in fibroblasts increases stromal component in AOM/DSS-induced lesions. A. Cellular proliferation in tumors was assessed by Ki67 staining and was similar in the control and ENG$^{Fib-/-}$ group. B. Apoptosis was determined by cleaved caspase 3 staining, which did not show a difference between the two groups. C. Total stroma content as quantified by vimentin staining and the abundance of activated fibroblasts as assessed by αSMA staining (D), proved to be increased after fibroblast-specific endoglin deletion. E. Total collagen deposition as measure by Sirius red was not affected by endoglin knock out in fibroblasts. Data represent 24/23 mice per group from two independent experiments, average number of positive pixels or average number of cells per high power field. *P≤0.05, ***P≤0.001.

Additionally, we assessed neutrophil infiltration in tumors using Ly6G expression. Flow cytometry showed that the percentage of Ly6G+ cells in the CD45+ population was strongly increased in the ENG$^{Fib-/-}$ group, when compared with control mice (Fig.4A). The number of Ly6G+ cells in adenomas was also assessed by IHC, and this analysis confirmed higher neutrophil infiltrate upon fibroblast-specific endoglin knock out (Fig. 4B). These data suggest that fibroblast-specific deletion of endoglin results in enhanced recruitment of immune cells, mainly macrophages and neutrophils.

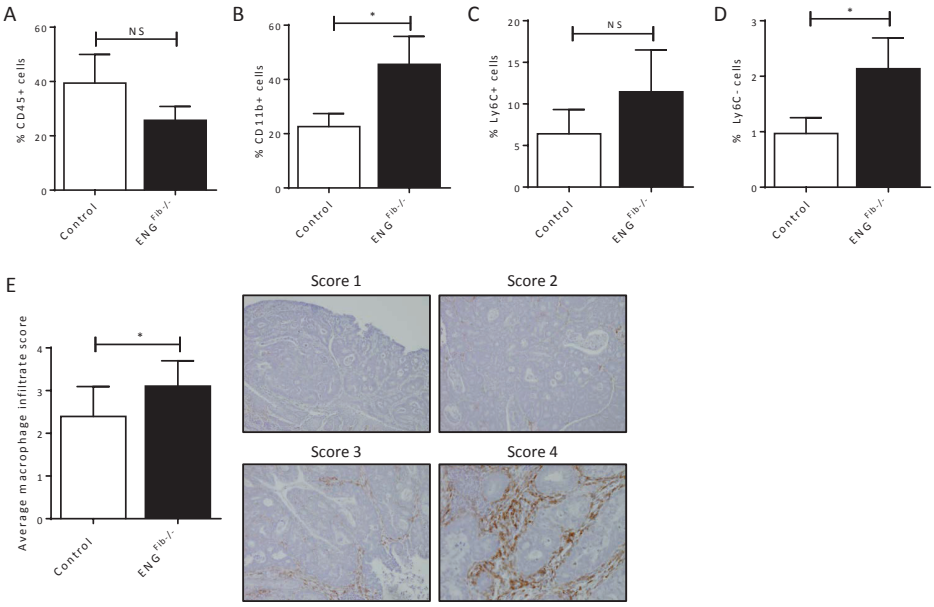


Figure 3. Increased macrophage recruitment to ENG$^{Fib-/-}$ lesions. Immune cell infiltrate in the lesions was determined using flow cytometry. CD45+ cells were gated out of the life cell population (A). Subsequently, CD11b+ cells were gated out of CD45+ (B). Next, F4/80 expressing cells were selected from the CD11b+ population. Using Ly6C expression, subdivision between Ly6C+ monocytes (C) and Ly6C- macrophages (D) was made, showing increased macrophage infiltration in ENG$^{Fib-/-}$ lesions. (n=3 tumors/group) E. The extent of macrophage infiltration was scored based on F4/80 IHC. Score 1; ≤5% stroma positive, score 2; 5-25% stroma positive, score 3; 25-50% stroma positive, score 4; ≥50% stroma positive. (n= 11-12 tumors/group). *P≤0.05.

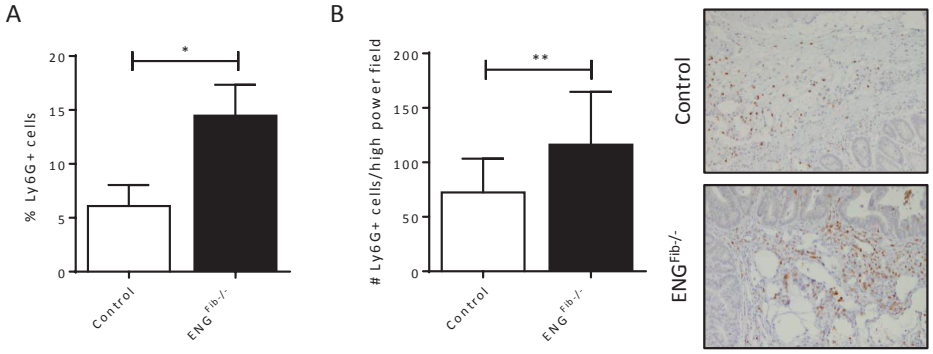


Figure 4. Fibroblast-specific endoglin deletion increases neutrophil recruitment. A. Flow cytometry showed increased neutrophil infiltrate in ENG$^{Fib-/-}$ lesions. Neutrophils were selected by gating for Ly6G from the CD45+/CD11b+ population. (n=3 tumors/group) B. IHC for Ly6G confirmed increased neutrophil influx in ENG$^{Fib-/-}$ lesions. Graph represents 24/23 mice per group from two independent experiments, average number of Ly6G+ cells. *P≤0.05, **P≤0.01.

Endoglin knock out in MEFs affects expression of inflammation-related genes *in vitro*

In order to confirm our *in vivo* observations that endoglin deletion in fibroblasts does not lead to increased epithelial proliferation in AOM/DSS induced lesions, *in vitro* proliferation was assessed. Since shRNA-mediated knockdown of endoglin in fibroblasts results in a lethal phenotype *in vitro* (chapter 7), we made use of murine embryonic fibroblasts (MEFs) from ENG*fl/fl* mice. Using lentiviral transduction, Cre recombinase was introduced in these cells, resulting in genetic deletion of endoglin (Fig. 5A). Conditioned medium (CM) from empty vector and endoglin knock out MEFs was prepared to assess paracrine signaling to mouse epithelial cells. CT26 and MC38 mouse CRC cells were stimulated with CM from either empty vector control or endoglin knock out MEFs and proliferation was measured. Over the course of three days, proliferation rates between non-stimulated cells, control CM or endoglin knock out CM stimulated cells were similar in both CT26 (Fig. 5B) and MC38 cells (Fig. 5C). This suggests that endoglin on fibroblasts does not directly affect epithelial tumor cell proliferation by secreting paracrine factors *in vitro,* confirming our *in vivo* findings. When we stimulated MC38 CRC cells with CM and assessed BMP signaling using the Bre-Luc reporter construct, response to control and endoglin knock out CM was similar and did not induce BMP signaling (Fig. 5D). Since MC38 cells were not responsive to TGF-β stimulation, we have used the mouse myoblast cell line C2C12 to determine effects of MEF CM on TGF-β signaling. Preliminary data show that TGF-β signaling was significantly induced by TGF-β stimulation and CM from control MEFs, whereas CM from endoglin knock out MEFs did not induce a response (Fig. 5E).

Next, considering the role of fibroblasts in immune cell recruitment by secretion of immunomodulatory factors, we assessed gene expression of these factors after endoglin knock out in MEFs. For this, RT-qPCR analysis was performed on empty vector control and endoglin knock out MEFs. Endoglin knock out in MEFs resulted in upregulation of IL-7 and MMP-9 (Fig. 6A) and downregulation of IL-8, TNFα, CXCR2, BMP-6, CXCL1 and all isoforms of TGF-β (Fig.6B). These data show that endoglin knock out in fibroblasts affects expression of TGF-β/BMP ligands and immunoregulatory factors.

Fibroblast-specific endoglin knock out changes immunoregulatory expression profiles *in vivo*

Based on the increased immune filtration in the lesions and differential expression of immunomodulatory factors in MEFs, we next assessed gene expression profiles of immune modulators in AOM/DSS-induced adenomas. As expected, endoglin expression was lower in lesions obtained from $ENG^{Fib-/-}$ mice, when compared with controls (Fig.7A). Additionally, as observed in MEFs, BMP-6 was expressed at lower levels in $ENG^{Fib-/-}$ lesions (Fig.7A). Although not significantly changed, we observed downregulation of CCL2, IL-2 and IL-7 in $ENG^{Fib-/-}$ lesions (Fig. 7B). Furthermore, mRNA expression of IL-6, IL-10, PDGFβ, IFNγ and SDF-1 was also decreased upon fibroblast-specific endoglin deletion (Fig. 7B). Moreover,

upregulation of the neutrophil attractant CXCL2 was observed in lesions from ENG$^{Fib-/-}$ mice (Fig. 7C), which is in agreement with increased neutrophil influx. Combined, these data show that fibroblast-specific endoglin deletion changes gene expression profiles of immunoregulatory genes *in vivo*.

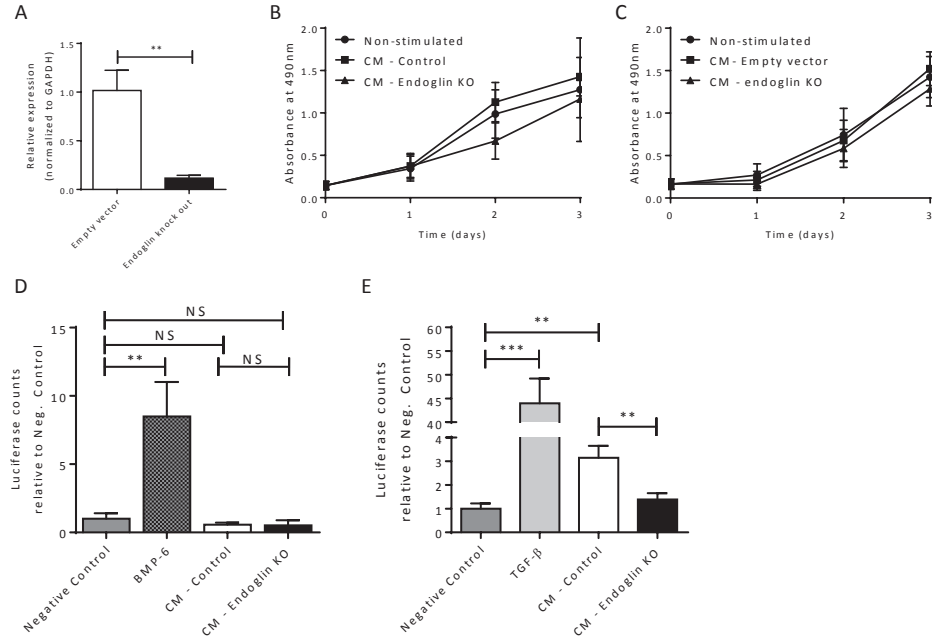


Figure 5. Conditioned medium from endoglin knock out MEFs lacks TGF-β-activating paracrine factors. A. MEFs were transduced with a Cre expressing lentivirus or empty vector control, significantly reducing endoglin expression after transduction with Cre recombinase. Graph represents mean of three independent experiments. Stimulation with conditioned medium from MEFs, either empty vector (CM – Control) or endoglin knock out (CM – Endoglin KO), did not affect tumor cell proliferation in CT26 (B) or MC38 cells (C). Graphs represent mean of three independent experiments performed in triplicate. D. Stimulation with CM from either empty vector or endoglin knock out MEFs did not enhance BMP signaling in a luciferase reporter assay. Graph represents mean of three experiments, performed in triplicate. E. TGF-β signaling was determined using the CAGA-Luc reporter construct, showing that CM from control MEFs induced TGF-β signaling in C2C12 cells, whereas CM from endoglin knock out MEFs did not. Graph represents single experiment, performed in triplicate. **P≤0.01, ***P≤0.001.

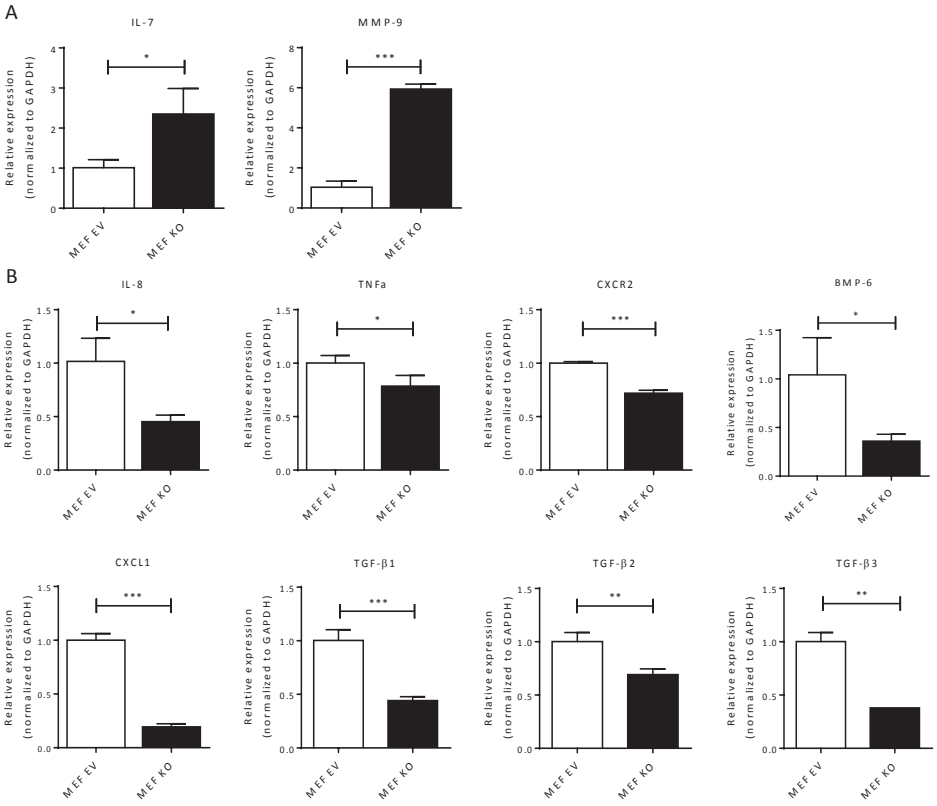


Figure 6. Endoglin knock out changes expression of chemokines in MEFs. RT-qPCR was performed to assess expression patterns of immunoregulatory genes. Endoglin knock out resulted in increased IL-7 and MMP-9 expression (A), while mRNA levels of IL-8, TNFα, CXCR2, BMP-6, CXLC1 and all TGF-β isoforms were decreased (E). Graphs represent data from three independent experiments. *P≤0.05, **P≤0.01, ***P≤0.001.

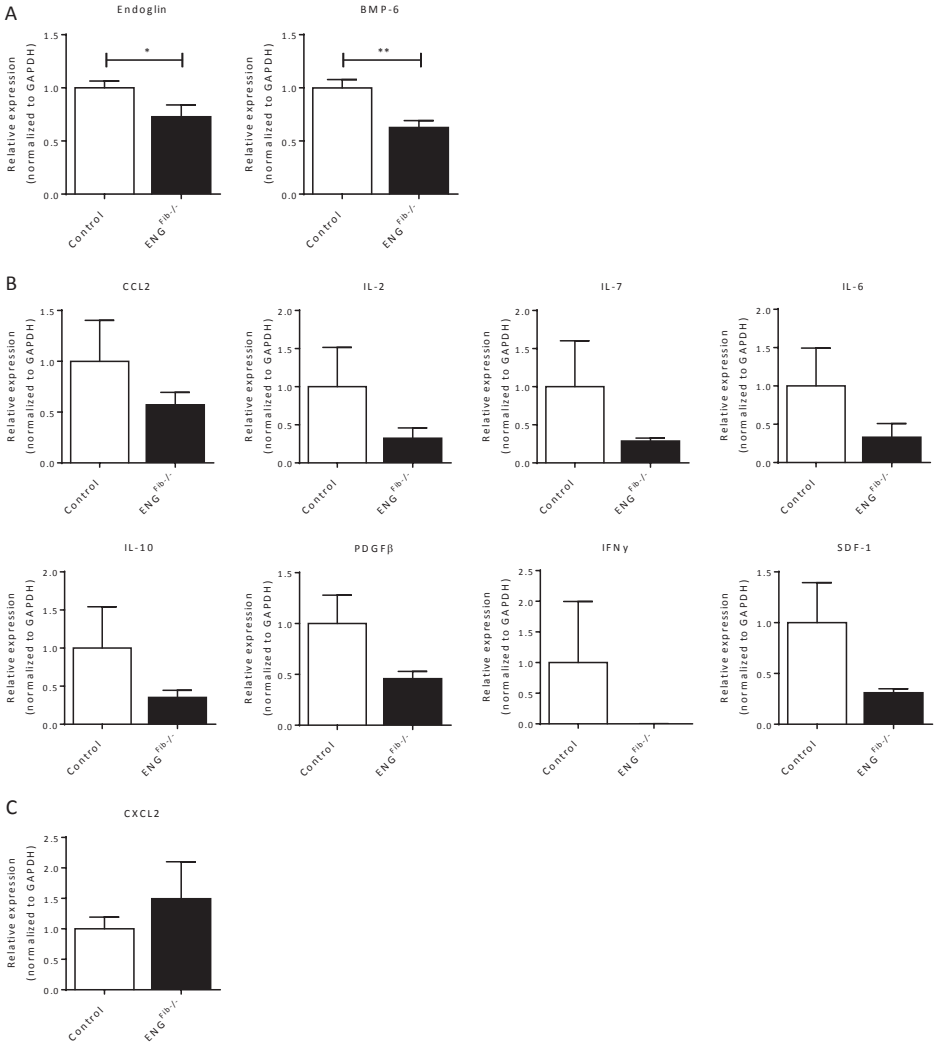


Figure 7. ENG$^{Fib-/-}$ lesions display changed expression of immunoregulatory genes. A. Lesions from ENG$^{Fib-/-}$ mice showed decreased expression of endoglin and BMP-6 at mRNA levels. B. A trend in decreased expression in the ENG$^{Fib-/-}$ group was observed for CCL2, IL-2, IL-7, IL-6, IL-10, PDGFβ, IFNγ and SDF-1. Expression of CXCL2 appeared to be increased (C). Graphs represent 9-5 lesions/group from two independent experiments. *P≤0.05, **P≤0.01.

Discussion

In this study we show that fibroblast-specific endoglin deletion increases tumorigenesis in a mouse model for inflammation-associated CRC. Fibroblast-specific endoglin deletion did not affect epithelial cell proliferation in adenomas, but resulted in stromal expansion and increased influx of macrophages and neutrophils into the lesions.

In intestinal homeostasis, the interplay between stroma and epithelium is crucial. When disrupted, imbalance in this interaction leads to decreased epithelial apoptosis or an increase in stem cell proliferation (*44, 45*), both resulting in spontaneous polyp formation. As shown by Beppu et al., inactivation of the BMP receptor type II (BMPRII) in intestinal stromal cells, increased epithelial cell proliferation and resulted in local polyp formation in the mouse colorectum (*46*). Similar effects have been observed for TGF-β receptor type II (TβRII) deletion in fibroblasts, resulting in neoplastic growth in the prostate and invasive stomach carcinoma *in vivo (47)*. Inactivation of BMPRII or TβRII leads to tumor formation within seven weeks in the colorectum or stomach, respectively (*46, 47*). In our study, we did not observe spontaneous tumor formation after endoglin deletion in fibroblasts during the 13-week experimental period. This observation is supported by our *in vitro* proliferation data, where stimulation with conditioned medium from endoglin knock out MEFs did not affect proliferation in two mouse CRC cell lines. These data suggest that endoglin expression on fibroblasts is not involved in regulating proliferation or apoptosis through paracrine factors.

Although no spontaneous tumors developed, fibroblast-specific endoglin deletion enhanced chemically-induced adenoma formation. One of the differences between lesions from the control and $ENG^{Fib-/-}$ group, was the increase of activated fibroblasts upon endoglin knock out. The $ENG^{Fib-/-}$ mice show worse survival due to complications of the high number of tumors. In CRC patients, the abundance of tumor stroma was reported to be prognostic for both overall and metastasis-free survival (*48*). This could imply that $ENG^{Fib-/-}$ lesions would be more aggressive and could have a higher metastatic potential. However, the severity of discomfort in our model does not allow for a prolonged experimental period, therefore tumor progression and metastatic spread could not be evaluated.

Contradictory to our observations reported here, our own recently published data show that treatment with the endoglin neutralizing antibody TRC105 reduced αSMA-positive tumor content in an *in vivo* breast cancer model (*49*). Additionally, a study in endoglin heterozygous mice reported that αSMA-positive content in prostate tumors was decreased, when compared to endoglin wild type mice (*50*). A major difference between aforementioned studies and our current research is that heterozygote endoglin deletion and TRC105 affect all cells expressing endoglin, which might explain the differential effects observed when compared to fibroblast-specific endoglin deletion.

In cancer, increased presence of activated fibroblasts in the stroma is known to regulate a TGF-β-induced fibrotic response, thereby stimulating tumor progression (*51, 52*). However, contradictory results have been published suggesting endoglin as either a

negative or a positive regulator of fibrosis (reviewed in (*53*)).The importance of endoglin in the fibrotic response was shown by decreased cardiac fibrosis in endoglin heterozygous mice (*54*). Moreover, in a mouse model for wound healing endoglin heterozygosity resulted in persistence of activated fibroblasts in wounds, which is related to fibrosis (*55*). This persistence could be an underlying mechanism for the increased presence of αSMA-positive cells in the $ENG^{Fib-/-}$ lesions in our study. Interestingly, during liver fibrosis, both cellular expression of endoglin and plasma levels of soluble endoglin, cleaved from the membrane by MMP-14 (*56*), are increased. These levels are even further enhanced in patients with both hepatocellular carcinoma (HCC) and liver cirrhosis, implying a role for endoglin in fibrosis-mediated disease progression (*57*). However, in our experiments total collagen deposition, as a marker for fibrosis, was similar in both groups. Yet, fibrosis is a highly TGF-β-dependent process (*58*). Therefore, the decreased expression of TGF-β after endoglin knock out (Fig. 6B) might partly explain the unaffected levels of collagen deposition in AOM/DSS-induced lesions.

Besides directly stimulating tumor progression, CAFs also produce cytokines and chemokines which can regulate the influx of immune cells. Immune cells can either inhibit or enhance tumor progression and metastasis (*59*). Heterozygous expression of endoglin has been shown to result in progression of acute inflammation to chronic inflammation after a single high dose of DSS, whereas in wild type mice inflammation was resolved after two weeks (*60*). Increased expression of vascular endothelial growth factor (VEGF) in endoglin heterozygous mice was proposed to be responsible for the persistent inflammation. Both *in vitro* and in AOM/DSS-induced adenomas, we did not observe a significant change in VEGF expression after endoglin knock out (Supplementary fig.S2). This discrepancy might be caused by the specific deletion of endoglin in fibroblasts, excluding other endoglin-positive VEGF producing cells, like endothelial cells.

Macrophages were shown to be one of the immune cells that have tumor promoting effects by, amongst others, releasing growth factors and proteases (*61-63*). Dependent on macrophage subset, these cells can be either tumor-promoting or -suppressive (*61, 64*). Infiltration of macrophages is generally correlated to worse prognosis in various types of cancer (*65-71*). However, in CRC high macrophage infiltration has been shown to correlate with a better prognosis, although macrophage subsets also play a differential role in this type of cancer (*72-76*). In our model, the ratio of M1/M2 macrophages could be informative regarding changed macrophage abundance in $ENG^{Fib-/-}$ adenomas and should therefore be assessed in future experiments.

Based on flow cytometry and IHC, we also observed increased neutrophil infiltration in lesions from $ENG^{Fib-/-}$ mice. Tumor promoting and pro-metastatic roles were reported for neutrophils, mainly via protease activation and secretion of pro-angiogenic factors (*77-81*). Moreover, neutrophils were shown to play a crucial role in AOM/DSS-induced tumorigenesis, since neutrophil depletion resulted in significantly reduced tumor initiation (*82, 83*). We observed a trend to increased CXCL2 expression in lesions from $ENG^{Fib-/-}$ mice, which is a

neutrophil attracting chemokine that is highly expressed by tumor stroma (*83*), thereby explaining increased neutrophil recruitment. Additionally, decreased IL-6 levels, as seen in tumors from ENG$^{Fib-/-}$ mice, were shown to result in increased neutrophil recruitment (*84*), posing another explanation for increased neutrophil infiltration. Both CXCL2 and IL-6 are highly produced by CAFs in CRC (*14*). Our data suggest opposite regulatory mechanisms of endoglin on fibroblasts in CXCL2 and IL-6 expression. Therefore, endoglin deletion in fibroblasts could simultaneously increase neutrophil attracting and reduce neutrophil repellant cytokines, thereby increasing overall neutrophil recruitment, as observed in our study.

BMP-6 was the only factor which was significantly downregulated in both endoglin knock out MEFs and ENG$^{Fib-/-}$ lesions. High levels of BMP-6 were shown to inhibit proliferation of breast cancer and myeloma cells *in vitro (85-87)* and appear to reduce aggressiveness of breast cancer cells in a zebrafish model (*88*). Lower levels of BMP-6 could therefore relieve inhibitory effects on epithelial cell proliferation and enhance tumor growth. However, since we did not observed effects on epithelial proliferation, BMP-6 does not seem to regulate neoplastic growth in our model.
Furthermore, BMP-6 reduces macrophage proliferation (*89*), suggesting that the decreased levels of BMP-6 in ENG$^{Fib-/-}$ lesions could release inhibition of macrophage proliferation, thereby posing an explanation for the increased presence of macrophages. However, mRNA levels do not directly reflect BMP-6 protein and activity levels, of which the latter is regulated by proteolytic cleavage (*90*), complicating definitive conclusions based on mRNA expression.
In summary, we show here that fibroblast-specific endoglin knock out results in enhanced initiation of neoplastic growth in a chemically-induced model for CRC. Based on our results we suggest that fibroblast-specific endoglin deletion results in changed expression profiles of immunoregulatory factors, thereby leading to increased immune cell recruitment and enhanced tumorigenesis. More specifically, due to the crucial role of neutrophils in AOM/DSS-induced tumors, we propose that increased neutrophil recruitment in ENG$^{Fib-/-}$ mice greatly contributes to chemically-induced neoplastic growth. To confirm that endoglin deletion in fibroblasts results in increased neutrophil-mediated tumorigenesis, experiments in ENG$^{Fib-/-}$ mice, in the context of neutrophil depletion should be performed.

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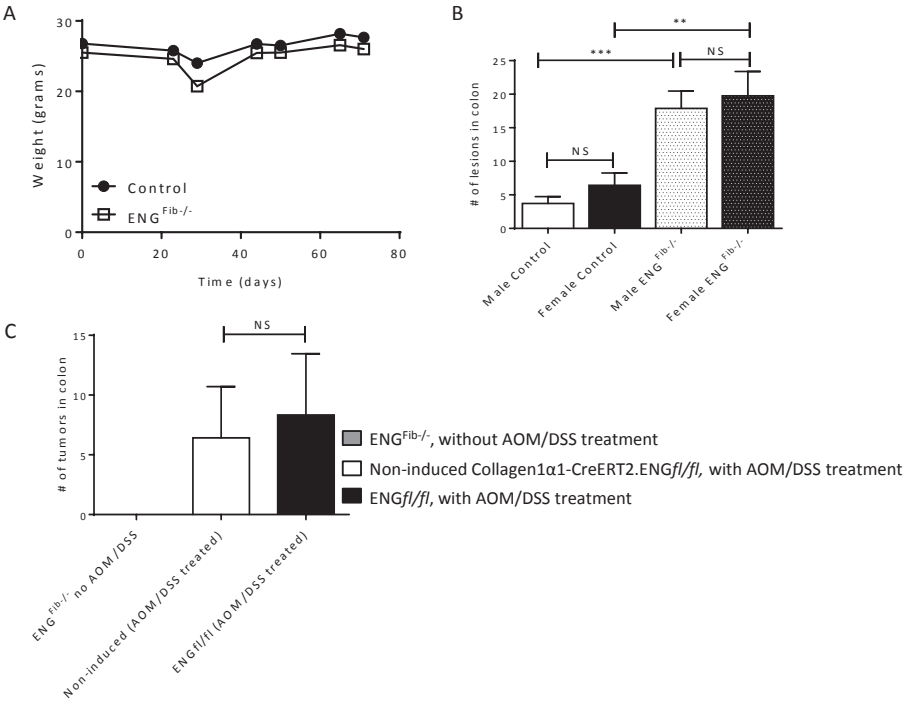
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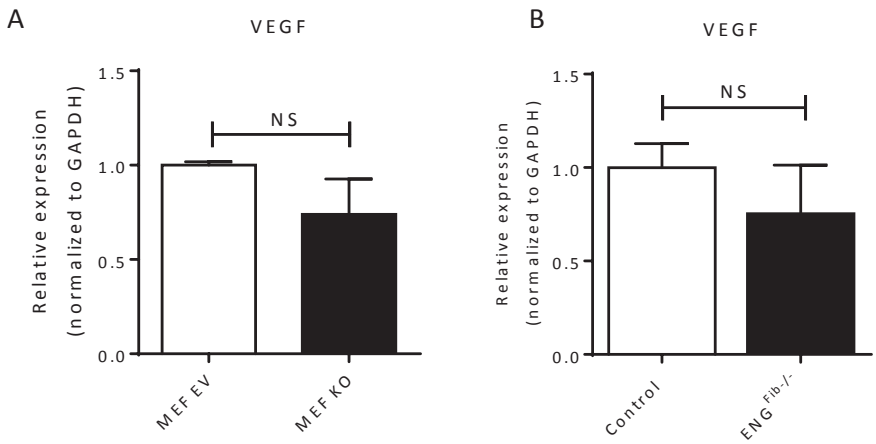
Supplementary data

Supplementary table S1 Primer sequences as used for RT-qPCR analysis

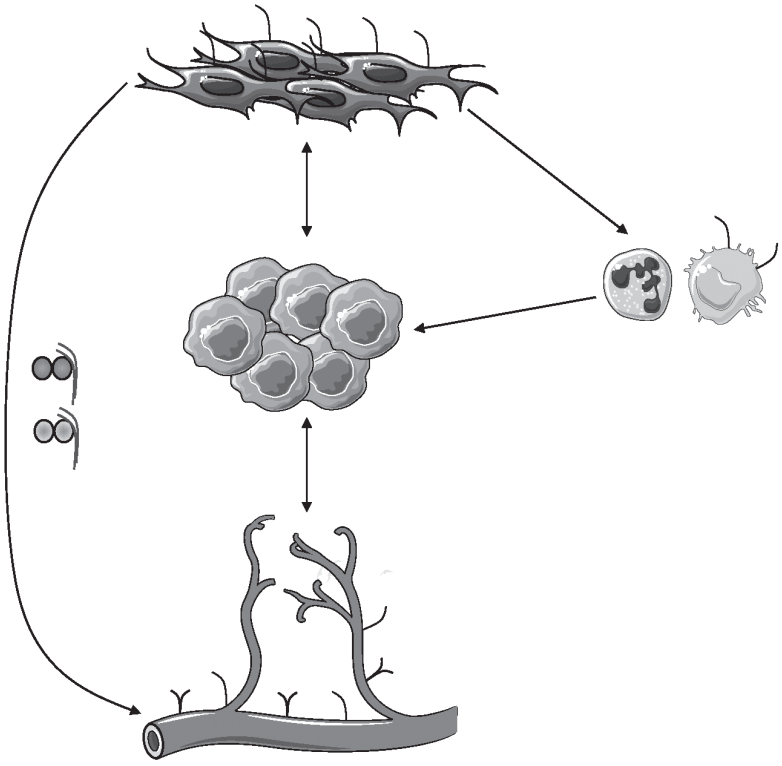
Gene	Forward	Reverse
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
Endoglin	CTTCCAAGGACAGCCAAGAG	GTGGTTGCCATTCAAGTGTG
IL-7	ACAGCCAGAGGAGTTGGAGA	GGGCTGACTGAAGTCTCAGG
MMP-9	TACAGGGCCCCTTCCTTACT	TGCCTGTGTACACCCACATT
IL-8	CGGCAATGAAGCTTCTGTAT	CCTTGAAACTCTTTGCCTCA
TNFα	TAGCCAGGAGGGAGAACAGA	TTTTCTGGAGGGAGATGTGG
CXCR2	AGCAGAGGATGGCCTAGTCA	TCCACCTACTCCCATTCCTG
BMP-6	AGCACAGAGACTCTGACCTATTTTTG	CCACAGATTGCTAGTTGCTGTGA
CXCL1	ACTGCACCCAAACCGAAGTC	TGGGGACACCTTTTAGCATCTT
CCL2	AGCACCAGCCAACTCTCACT	CGTTAACTGCATCTGGCTGA
IL-2	AAGCTCTACAGCGGAAGCAC	ATCCTGGGGAGTTTCAGGTT
IL-6	GTATGAATAACGATGATGCACTTG	ATGGTACTCCAGAAGACCAGAGGA
IL-10	CCAGGGAGATCCTTTGATGA	AACTGGCCACAGTTTTCAGG
PDGF-β	TTTGGAGACTTGGGCTGGGA	ACGGACCCCCAGATCAGAA
IFNγ	GCTTTAACAGCAGGCCAGAC	GGAAGCACCAGGTGTCAAGT
SDF-1	GAAAGGAAGGAGGGTGGCAG	TCCCCGTCTTTCTCGAGTGT
CXCL2	ATCCAGAGCTTGAGTGTGACG	GTTAGCCTTGCCTTTGTTCAG
TGF-β1	CAACAATTCCTGGCGTTACC	TGCTGTCACAAGAGCAGTGA
TGF-β2	CCGCCCACTTTCTACAGACCC	GCGCTGGGTGGGAGATGTTAA
TGF-β3	GTTTGCAGCATTTGTGATCG	TGCTCTGAGTGCTCCCTATG
VEGF	ACCAGCGAAGCTACTGCCGT	TAACTCAAGCTGCCTCGCCT



Supplementary figure S1. A. Mouse weights dropped during DSS supplementation, but recovered during the two weeks on normal drinking water. B. The number of lesions observed in the AOM/DSS model is independent of animal sex, in both the control and ENG$^{Fib-/-}$ group. C. Fibroblast-specific endoglin deletion without AOM/DSS treatment did not induce neoplastic growth over the course of 13 weeks (n=8). ENG*fl/fl* mice, which received tamoxifen, showed similar number of lesions as non-induced collagen1α1-CreERT2. ENG*fl/fl* mice after AOM/DSS (n=9).



Supplementary figure S2. A. VEGF expression was determined by RT-qPCR and expression levels were not changed upon endoglin knock out in MEFs. B. VEGF mRNA levels were not changed in AOM/DSS-induced adenomas when endoglin was deleted in fibroblasts. Graph represents 9-5 lesion/group from two independent experiments.



Chapter 9

General discussion

Summarizing discussion

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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

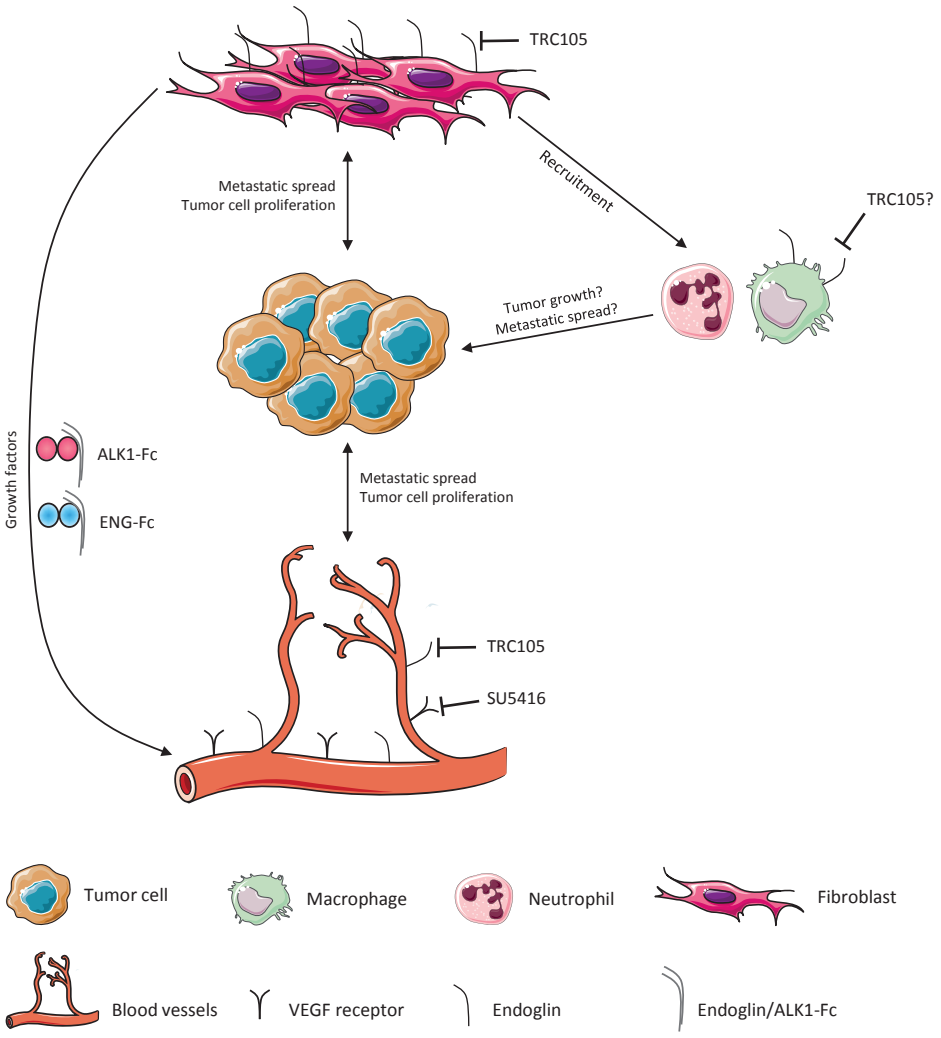


Figure 1. The studied interactions between tumor and stromal cells and interfering therapeutic strategies. Epithelial tumor cells interact with endothelial cells to induce tumor angiogenesis, supplying the tumor with nutrients and oxygen and providing a route of dissemination for metastatic spread. CAF-tumor 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.

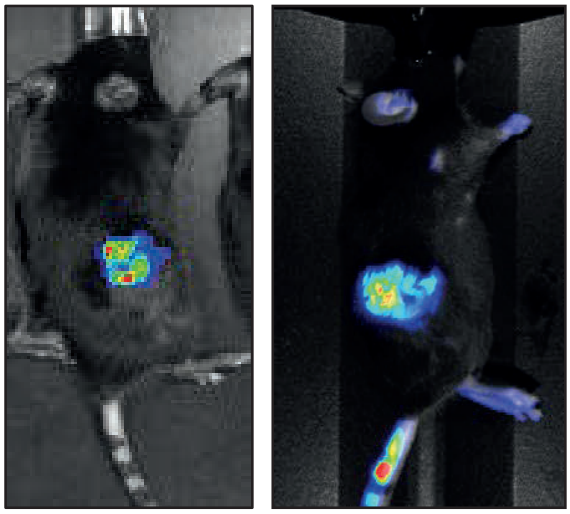


Figure 2. NIRF imaging using TRC105 results in accurate tumor localization. Luciferase-expressing mouse CRC cells were subcutaneously transplanted and clearly visualized by bioluminescent imaging. NIRF imaging using fluorescently labeled TRC105 showed an overlapping signal with bioluminescence, indicating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

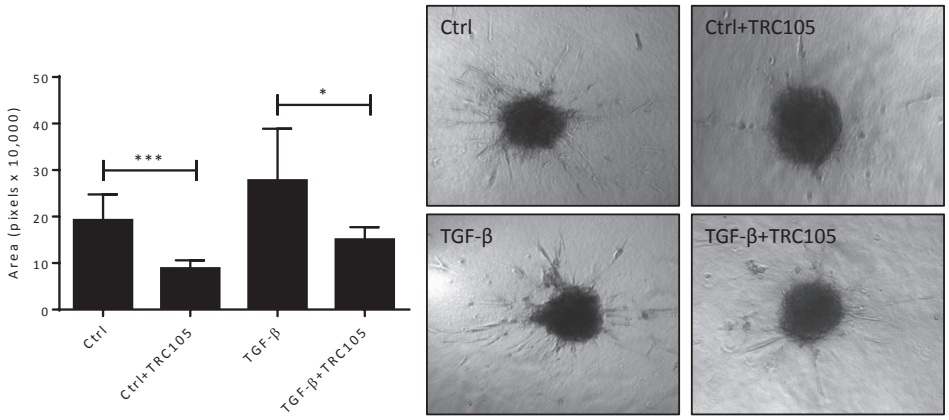


Figure 3. TRC105 indirectly inhibits TGF-β-mediated CAF invasion. Spheroids of CAFs were embedded in a collagen-I matrix and invasion was assessed after two days. Treatment of the cells with TRC105 decreased both FCS- and TGF-β-induced CAF invasion. Since TRC105 does not directly inhibit TGF-β binding 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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Chapter 10

Nederlandse samenvatting voor niet-ingewijden

1. Algemene introductie

Ondanks de grote vooruitgang in de afgelopen decennia, is kanker nog steeds een van de belangrijkste doodsoorzaken in de Westerse wereld. Bij de meeste vormen van kanker is niet de primaire tumor, maar de vorming van uitzaaiingen (metastases) de voornaamste oorzaak van patiëntensterfte. Dat is ook het geval in borst- en dikke darmkanker. Voor deze twee vormen van kanker wordt op basis van risico inschatting bepaald of patiënten baat hebben bij aanvullende anti-kanker therapieën. De beslissing hierover wordt in het huidige systeem voornamelijk gebaseerd op de stadiëring van de primaire tumor. Hierbij wordt bekeken of de tumor ingroeit in het gezonde weefsel en of de tumor al is uitgezaaid en zo ja, hoe ver. Voor zowel borst- als darmkanker geldt dat patiënten die zich presenteren zonder uitzaaiingen, niet gezien worden als risicogroep voor het ontwikkelen van uitzaaiingen. Op basis hiervan ondergaan deze patiënten alleen chirurgische verwijdering van de tumor en geen aanvullende behandelingen. Ondanks de hoge overlevingskans in deze patiëntengroep is er een gedeelte van de patiënten (~30%) dat uiteindelijk toch uitzaaiingen krijgt en hieraan zelfs overlijdt. Dit benadrukt dat er een betere selectie nodig is om deze patiënten te identificeren en om uitzaaiingen te voorkomen en behandelen.

2. Tumor micro-omgeving

De basis voor iedere tumor ligt in het optreden van veranderingen in het DNA (genetische mutaties) in lichaamscellen, waardoor deze cellen ongeremd kunnen vermenigvuldigen en de groei zelfs gestimuleerd wordt. De ontregeling, van deze normaal strak gereguleerde processen, leidt uiteindelijk tot tumorvorming. Sinds men weet dat genetische mutaties de basis zijn voor tumorgroei zijn er veel behandelingen ontwikkeld die gericht zijn op het aanvallen van de kankercellen. Doordat de mutaties in tumorcellen continu en op hoge snelheid optreden gedurende het groeien van de tumor worden veel tumoren snel ongevoelig (resistent) tegen deze behandelingen. Dit leidt vervolgens tot hernieuwde groei van de tumor, vaak een verminderde kwaliteit van leven of uiteindelijk zelfs overlijden. Meer recentelijk is gebleken dat de directe omgeving van de tumorcellen, de tumor micro-omgeving (TME), erg belangrijk is voor tumorgroei en de vorming van uitzaaiingen. Deze micro-omgeving bestaat uit cellen die de tumor ondersteunen, waaronder cellen die bloedvaten vormen zodat de tumor genoeg zuurstof en voedingsstoffen ontvangt. Andere belangrijke cellen in deze omgeving zijn ontstekingscellen en de steuncellen van de tumor, kanker geassocieerde fibroblasten (CAFs) genaamd. In zowel darm- als borstkanker is aangetoond dat de totale omvang van deze tumor micro-omgeving, die per tumor verschilt, een voorspellende factor is voor de overlevingskans van de patiënten hoe meer cellen in de micro-omgeving, hoe groter de kans op overlijden. Sinds het identificeren van de stimulerende rol van de micro-omgeving in tumorprogressie en de van vorming

uitzaaiingen zijn er therapeutische strategieën ontwikkeld gericht tegen de tumor micro-omgeving.
Cellen kunnen onderling met elkaar communiceren door middel van signaleringsroutes. Dit houdt in dat wanneer de ene cel een signaal geeft, dat wordt doorgegeven door middel van deze signaleringsroutes naar een andere cel. Een van de signaleringsroutes die een belangrijke rol speelt in alle componenten van de micro-omgeving is de Transforming Growth Factor-β (TGF-β) route. Door deze prominente rol wordt de TGF-β signalering gezien als een goed aangrijpingspunt voor behandelingen tegen kanker.

De TGF-β signaleringsroute is van essentieel belang tijdens de ontwikkeling en gedurende het normaal functioneren van het menselijk lichaam. Zoals gezegd speelt TGF-β ook een belangrijke rol in kanker. Waar TGF-β initieel tumorgroei remt, functioneert het in verder gevorderde tumoren juist als een stimulerende factor. TGF-β is een van de vele factoren uit de TGF-β familie, waar ook Bone Morphogenic Proteins (BMPs) deel van uitmaken. Om signalen van de ene naar de andere cel op een juiste manier door te geven, is een geavanceerd systeem noodzakelijk. Dit systeem is het beste te vergelijken met het kinderspeelgoed waar een specifieke gevormde blokken alleen door een specifiek gat door het deksel passen. Wanneer het blokje (signaleringsfactor) niet past op het gat (een receptor), zal het niet door het deksel passen en dus geen signaal door kunnen geven aan de ontvangende cel. De factoren uit de TGF-β signaleringsfamilie (blokjes) hebben allemaal een specifieke vorm en maken daarom gebruik van specifieke receptoren op de cellen (gaatjes in het deksel) om hun functie uit te oefenen. Wanneer een cel een benodigde receptor niet heeft, zal deze dus geen signalen kunnen ontvangen via die specifieke factor. De voor dit proefschrift belangrijke receptoren (gaten in het deksel) voor TGF-β en BMP signalering zijn ALK1 (onderzocht in **hoofdstuk 4**) en endoglin (onderzocht in **hoofdstuk 5, 7 en 8**). Beide receptoren kunnen binden aan familieleden van de TGF-β signaleringsfamilie, maar hebben specificiteit voor andere signaleringsfactoren (blokjes). De aan- of afwezigheid van deze receptoren op een cel stuurt het effect van de signaleringsfactoren in een bepaalde richting. Zo is bijvoorbeeld de aanwezigheid van endoglin op bloedvatcellen betrokken bij het sturen van TGF-β signalering in de richting van bloedvatformatie (angiogenese). Wanneer endoglin niet aanwezig is op de bloedvatcellen zal TGF-β signalering leiden tot remming van bloedvatformatie. Naast de aanwezigheid van endoglin op bloedvatcellen, is endoglin ook aangetoond op specifieke tumorcellen, sommige ontstekingscellen en op CAFs (**hoofdstuk 7)**. De functies die endoglin in deze cellen vervult zijn cel-specifiek.
Omdat zowel endoglin als ALK1 veel voorkomen op bloedvatcellen is er veel onderzoek gedaan naar het gebruiken van deze receptoren om bloedvatvorming in tumoren te remmen. Deze strategieën worden ook klinisch toegepast voor de behandeling van verschillende vormen van kanker. Dit proefschrift richt zich op de rol van TGF-β en BMP signalering in de tumor micro-omgeving tijdens tumor progressie en uitzaaiing. Daarnaast wordt de mogelijkheid onderzocht om de signalering via ALK1 of endoglin te onderbreken voor potentieel gebruik als behandeling tegen kanker.

3. Resultaten

In **hoofdstuk 3**, een samenvatting van 161 eerder gepubliceerde artikelen, wordt de potentie van de receptor endoglin als aangrijpingspunt voor kankerbehandelingen uitgebreid besproken. Doordat endoglin in grote hoeveelheden aanwezig is op nieuwgevormde bloedvaten, zoals dat in een tumor gebeurt en verder op weinig andere plekken in een gezond lichaam, maakt het een zeer geschikt aangrijpingspunt om deze bloedvaten aan te vallen. Daarnaast zorgt de lokalisatie van endoglin, aan de binnenkant van de bloedvaten, ervoor dat de receptor gemakkelijk toegankelijk is voor medicatie toegediend via bloed. Een voorbeeld van zulke medicatie is het toedienen van antilichamen. Wanneer we de vergelijking met de blokkendoos doortrekken zijn antilichamen stoffen die binden aan de receptor (het gat in de deksel) of de signaleringsfactor (het blokje) waardoor het blokje niet meer door het gat in de deksel past. Hierdoor zal de geïnitieerde communicatie geen doorgang kunnen vinden en wordt het beoogde effect niet bereikt. Naast behandelen, is ook de beeldvorming van een tumor van groot klinisch belang en kan deze techniek helpen bij complete chirurgische verwijdering. Hierdoor vermindert het risico op terugkeer van de tumor en het ontstaan van uitzaaiingen. Het gebruik van een oplichtend antilichaam dat bindt aan endoglin kan, door de specifieke expressie, helpen om op een efficiënte manier de tumor in beeld te brengen. Endoglin werkt in de signalering vaak samen met ALK1, een andere TGF-β receptor. De mogelijkheid om ALK1 te gebruiken als aangrijpingspunt om bloedvatgroei in tumoren te remmen, werd onderzocht in **hoofdstuk 4**. Deze studie beschrijft dat het remmen van ALK1 signalering met een antilichaam resulteerde in verminderde tumorbloedvatgroei in muismodellen voor verschillende vormen van kanker. Door het remmen van de bloedvatgroei verbeterde de kwaliteit van de resterende tumorbloedvaten. Hierdoor beschikte de tumoren nog steeds over genoeg zuurstof en voedingsstoffen waardoor de grootte van de tumoren niet werd beïnvloed. Deze verbeterde bloedvatkwaliteit en bloedtoevoer kan zorgen voor een betere bereikbaarheid van de tumor voor via het bloed toegediende chemotherapie. Het remmen van ALK1 in combinatie met chemotherapie zorgde voor verminderde tumorgroei in de diverse, in dit hoofdstuk onderzochte kankersoorten (borstkanker, hoofd- en halstumoren en huidkanker). Deze combinatietherapie wordt momenteel verder onderzocht in het laboratorium, maar ook in de kliniek. De effecten van het remmen van endoglin signalering op bloedvatgroei in borstkanker worden besproken in **hoofdstuk 5**. In deze studie werd onderzocht of het gelijktijdig remmen van twee signaleringsfactoren die bloedvatgroei stimuleren, VEGF en endoglin, zou leiden tot verminderde borsttumorgroei. In zowel celkweekexperimenten als in zebravissen was de gecombineerde behandeling efficiënter in het remmen van bloedvatgroei dan beide behandelstrategieën apart. In een muismodel voor borstkanker verminderde de combinatie behandeling de hoeveelheid tumorbloedvaten, maar de tumorgrootte veranderde niet. Opvallend genoeg was het aantal uitzaaiingen sterk verminderd na behandeling met een antilichaam tegen endoglin, TRC105 genaamd. Dit was een

zeer belangrijke bevinding voor het bestrijden van uitzaaiingen, ook in de kliniek. Opvallend was dat naast het effect op de bloedvaten van de tumor ook het aantal CAFs, de steuncellen, in de tumoren waren verminderd na anti-endoglin behandeling. De aanwezigheid van een grote hoeveelheid CAFs in de tumor is in patiënten gerelateerd aan een ernstiger ziekteverloop. Tezamen duiden deze resultaten erop dat het remmen van endoglin zowel tumor bloedvatvorming als CAFs remt en op deze manier een groot effect heeft op het uitzaaien van borstkanker. Deze resultaten werden verder onderzocht in de studies beschreven in **hoofdstuk 6 en 7**.

In **hoofdstuk 6** werd de stimulerende rol van CAFs in het ingroeien van tumorcellen in het omliggende gezonde weefsel aangetoond. Wanneer CAFs in celkweekexperimenten behandeld werden met stoffen die uitgescheiden worden door tumorcellen, zogenaamd geconditioneerd medium, leidde dit tot meer TGF-β signalering in de CAFs en hyperactivatie van CAFs. Deze twee processen spelen een belangrijke rol in de ingroei van tumorcellen in het gezonde weefsel en tijdens de vorming van uitzaaiingen. Samenvattend leidde de interactie tussen tumorcellen en CAFs tot een TGF-β afhankelijke signalering in een zichzelf versterkende vicieuze cirkel, die stimulerend werkte voor tumorgroei en –progressie. Het aanwezig zijn van endoglin op CAFs en de rol op deze cellen in darmkanker wordt beschreven in **hoofdstuk 7**. In dit hoofdstuk werd aangetoond dat naast endotheelcellen ook specifieke CAFs in het gedeelte waar de tumor het gezonde weefsel binnendringt endoglin hebben. Deze endoglin-positieve CAFs werden ook teruggevonden in uitzaaiingen van darmkanker, zowel in lymfeklieren als in uitzaaiingen in de lever. Dit wijst erop dat CAFs die signalen kunnen ontvangen via endoglin een stimulerende rol spelen in de vorming van uitzaaiingen. Daarnaast bleek het aanwezig zijn van endoglin op CAFs in vroeg stadium darmkankerpatiënten, met een laag risico op uitzaaiingen, voorspellend te zijn voor het uitzaaien van de tumor. Hierdoor zouden deze "hoog risico" patiënten beter geïdentificeerd kunnen worden. In muizen met uitzaaiende darmkanker verminderde het aantal uitzaaiingen in de lever wanneer signalering via endoglin geremd werd. Deze resultaten wijzen op een belangrijke rol voor endoglin op CAFs in het proces van darmkankeruitzaaiing. De remming van signalering via endoglin op CAFs zou een aangrijpingspunt kunnen bieden voor nieuwe behandelingen.

Omdat endoglin op CAFs een belangrijke rol lijkt te spelen in darmkankeruitzaaiing werd een muis gegenereerd waarin, op ieder gewenst moment, endoglin uitgeschakeld kan worden specifiek in de CAFs. Wanneer we weer naar de vergelijking kijken met de blokkendoos, zorgt dit er voor dat het gat in de deksel (endoglin) helemaal verdwijnt. Hierdoor kunnen de blokken die hier normaal doorheen passen niet meer in de doos terecht komen en dus geen signaal doorgeven. Na het uitschakelen van endoglin werd in deze muizen darmkanker veroorzaakt. Vervolgens werd onderzocht of CAF-specifieke uitschakeling van endoglin een effect heeft op tumorvorming en uitzaaiingen, zoals beschreven in **hoofdstuk 8**. Tot onze verbazing ontstonden er in muizen waarin endoglin was uitgeschakeld meer vroeg stadium tumoren (adenomen) dan in controledieren. Wanneer de tumoren gedetailleerd onderzocht werden, bleek dat er meer CAFs aanwezig

waren wanneer endoglin uitgeschakeld was. Daarnaast, was ook de aanwezigheid van twee typen ontstekingscellen toegenomen na endoglin uitschakeling.
Meer gedetailleerde experimenten lieten zien dat na het uitschakelen van endoglin, meer factoren worden gemaakt die ontstekingscellen aantrekken. Deze verandering zou een verklaring kunnen zijn van de verhoogde aanwezigheid van ontstekingscellen in de tumoren. Ontstekingscellen spelen een grote rol in het ontstaan van tumoren en hun verdere groei, zowel stimulerend als remmend. Gebaseerd op deze data is het noodzakelijk verdere studies te doen naar de rol van endoglin op de steuncellen van de tumor, de CAFs, in het aantrekken van ontstekingscellen.

4. Conclusies

De studies in dit proefschrift bevestigen de uitgebreide interactie tussen verschillende celtypen in de tumor micro-omgeving en hoe deze interacties klinisch relevant kunnen zijn voor het ontwikkelen van nieuwe kankerbehandelingen. Het gebruiken van de receptoren ALK1 of endoglin op bloedvaten als aangrijpingspunt voor behandelingen kan leiden tot een verbeterde efficiëntie van bestaande chemotherapie. Daarnaast spelen CAFs een belangrijke rol in tumorgroei en -uitzaaiing en lijkt de aanwezigheid van endoglin op deze cellen daar een sleutelrol in te spelen. Als laatste leidt de uitschakeling van endoglin op CAFs tot een toename van het aantal tumoren in een muismodel voor darmkanker. Deze tumoren laten zowel een verhoogd percentage CAFs zien als een verhoogde aanwezigheid van ontstekingscellen.

5. Klinische relevantie

Uit de in dit proefschrift beschreven studies blijkt dat TGF-β signalering in de tumor micro-omgeving een belangrijke rol speelt in tumorgroei en uitzaaiing. Het remmen van deze signalering lijkt efficiënt bereikt te kunnen worden door ALK1 of endoglin als aangrijpingspunt te gebruiken. Het remmen van ALK1 signalering wordt in kankerpatiënten onderzocht om bloedvaten in de tumor aan te vallen. Daarnaast wordt de therapeutische remming van ALK1 signalering in combinatie met chemotherapie ook in klinische studies bestudeerd. Het gebruik van het anti-endoglin antilichaam TRC105 wordt momenteel beoordeeld in klinische studies. Hierin wordt gekeken naar het effect op tumorbloedvatgroei en de mogelijke combinatie met chemotherapie.

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Curriculum vitae

Madelon Paauwe werd geboren op 12 januari 1986 te Leiden. Na het behalen van het HAVO diploma op het Adelbert College te Wassenaar in 2003, gevolgd door de propedeuse fysiotherapie aan de Hogeschool Leiden in 2004, werd in 2005 het VWO diploma voor biologie, wis-, natuur- en scheikunde behaald aan het ROC Leiden. In 2006 werd gestart met de studie Biomedische Wetenschappen aan de Universiteit Leiden/Leids Universitair Medisch Centrum. De master Biomedical Sciences werd in 2011 afgerond met een afstudeerstage bij het Nederlands Kanker Instituut in Amsterdam waar, onder leiding van Dr. Rogier Rooswinkel en Prof. Dr. Jannie Borst, onderzoek werd gedaan naar de interactie tussen pro- en anti-apoptotische eiwitten. In 2012 werd Madelon aangesteld als promovenda op de afdeling Moleculaire Celbiologie van het Leids Universitair Medisch Centrum om de rol van endoglin op tumor geassocieerde fibroblasten in kanker te onderzoeken in de onderzoeksgroep van Prof. Dr. Peter ten Dijke, onder begeleiding van Dr. Lukas Hawinkels. Na drie jaar verhuisde de onderzoeksgroep van Dr. Hawinkels naar de afdeling Maag-, Darm- en Leverziekten van het Leids Universitair Medisch Centrum, waar het project werd voortgezet gezamenlijk met de onderzoeksgroep van Prof. Dr. James Hardwick. Na afronding van dit onderzoek is Madelon als Post-Doc aangesteld op de afdeling Trombose en Hemostase van het Leids Universitair Medisch Centrum, onder supervisie van Prof. Dr. Henri Versteeg.

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- I don't stop when I'm tired, I stop when I'm done –
Unknown