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

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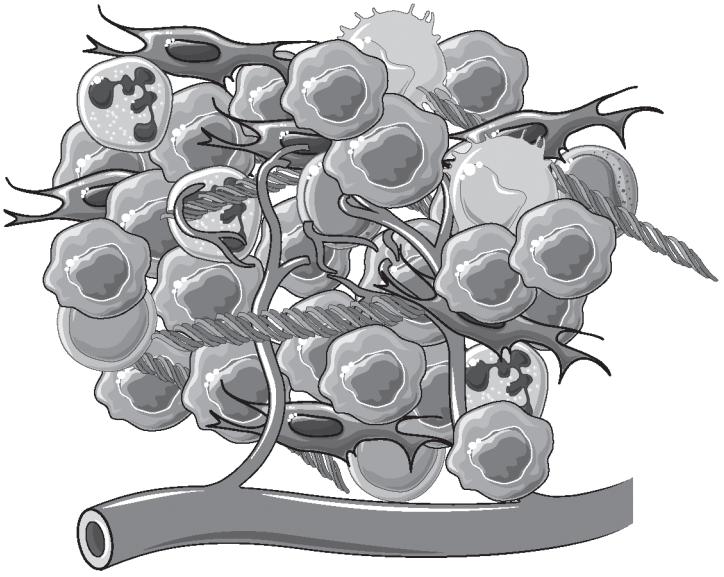

Chapter 1

General introduction

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*).

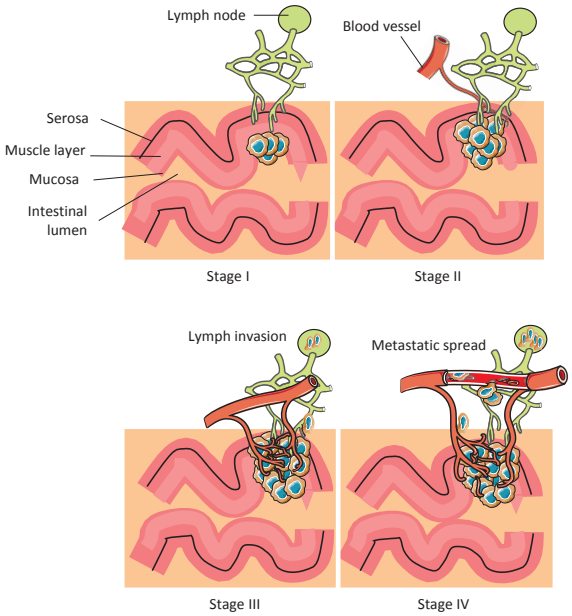

Figure 1. Schematic representation of the different stages of CRC development. Stage-I is characterized by the formation of a small tumor mass which invades through the intestinal mucosa into the muscle layer. During progression to stage-II CRC, angiogenesis is induced and tumor cells start to invade 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.

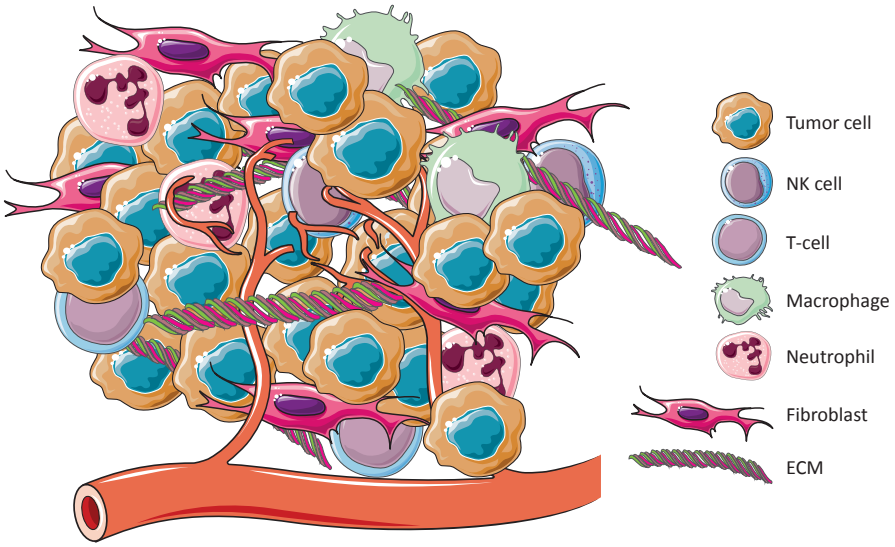

Figure 2. The tumor microenvironment. Epithelial tumor cells are surrounded by the tumor microenvironment which exists of endothelial cells, fibroblasts, extracellular matrix and a variety of infiltrated immune cells.

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.

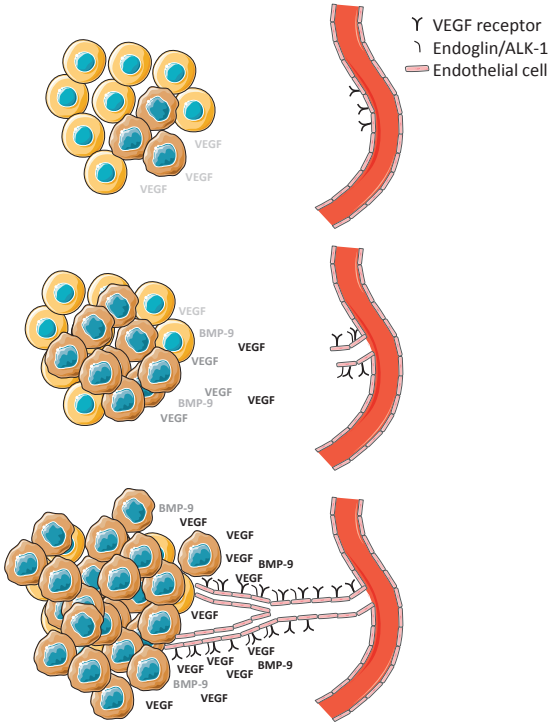

Figure 3. The process of tumor angiogenesis. In response to hypoxia, due to tumor growth, tumor cells start to secrete pro-angiogenic factors like VEGF and BMP-9. Endothelial cells sense increased concentrations of VEGF or BMP-9 with the VEGF receptor II or endoglin/ALK1, respectively. As a result, endothelial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

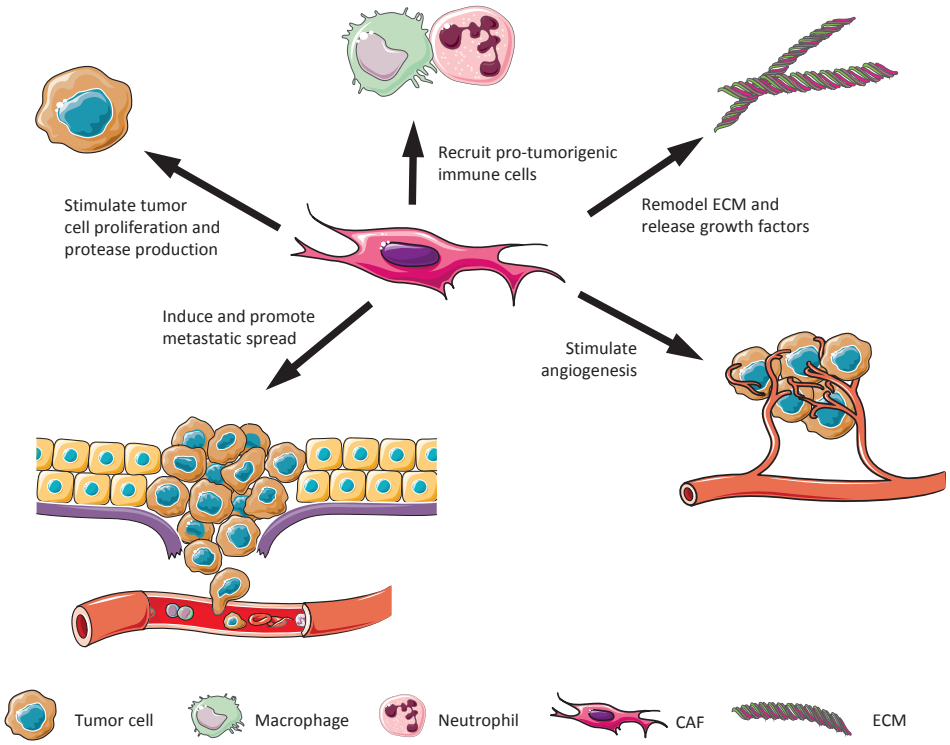

Figure 4. Pro-tumorigenic interactions of CAFs. CAFs stimulate tumor progression and metastasis by interacting with all cell types in the TME and by remodeling the extracellular matrix (ECM).

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.

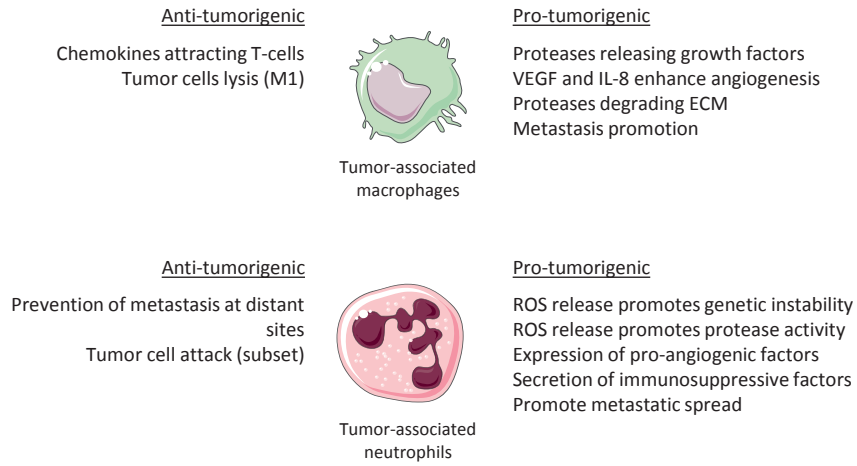

Figure 5. Pro- and anti-tumorigenic properties of macrophages and neutrophils. Macrophages and neutrophils are often observed in solid tumors and were reported to have both pro- and anti-tumor effects.

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.

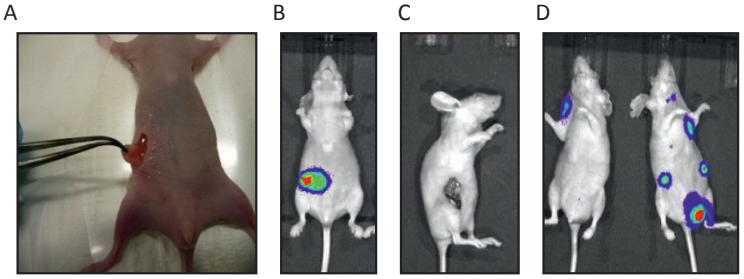

Figure 6. Orthotopic breast cancer model. Mouse breast cancer cells are transplanted to the mammary fat pad (A), and tumor growth is monitored by bioluminescence (B). For the resection model, the primary tumor was resected after four weeks (C) and metastatic spread was followed over time (D).

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.

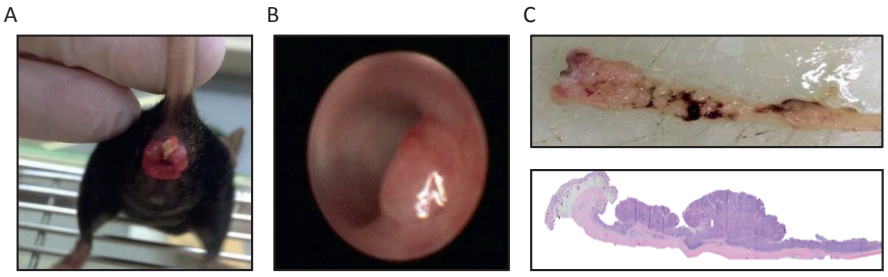

Figure 7. Chemically-induced mouse model for CRC. Colorectal tumor growth is induced by AOM/DSS treatment. During DSS cycles mice suffer from colitis, which, in combination with tumor growth, results in rectal blood loss and occasionally rectal prolapse (A). Tumor growth in this model can be monitored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

References

1. Cancer Research UK. (2016).
2. World Health Organization. (2015).
3. National Center of Health Statistics, Leading Causes of Death. (2015).
4. L. A. Torre *et al.*, Global cancer statistics, 2012. *CA Cancer J. Clin* **65**, 87-108 (2015).
5. S. McGuire, World Cancer Report 2014. Geneva, Switzerland: World Health Organization, International Agency for Research on Cancer, WHO Press, 2015. *Adv Nutr* **7**, 418-419 (2016).
6. American Joint Committee on Cancer (AJCC), *AJCC cancer staging handbook from the AJCC cancer staging manual. 6th edition*. G. F. P. D. F. I. F. A. B. C. H. D. M. M, Ed., (Springer-Verlag, 2002).
7. X. Dai *et al.*, Breast cancer intrinsic subtype classification, clinical use and future trends. *Am J Cancer Res* **5**, 2929-2943 (2015).
8. C. M. Perou *et al.*, Molecular portraits of human breast tumours. *Nature* **406**, 747-752 (2000).
9. T. Sorlie *et al.*, Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* **100**, 8418-8423 (2003).
10. C. Sotiriou *et al.*, Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci U S A* **100**, 10393-10398 (2003).
11. C. M. Ho-Yen, J. L. Jones, S. Kermorgant, The clinical and functional significance of c-Met in breast cancer: a review. *Breast Cancer Res* **17**, 52 (2015).
12. C. S. Vallejos *et al.*, Breast cancer classification according to immunohistochemistry markers: subtypes and association with clinicopathologic variables in a peruvian hospital database. *Clin Breast Cancer* **10**, 294-300 (2010).
13. T. Sorlie *et al.*, Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* **98**, 10869-10874 (2001).
14. R. Haque *et al.*, Impact of breast cancer subtypes and treatment on survival: an analysis spanning two decades. *Cancer Epidemiol Biomarkers Prev* **21**, 1848-1855 (2012).
15. L. H. Sobin, C. Wittekind, *TNM Classification of Malignant Tumors. 6th ed.*, (NY Wiley-Liss, 2002).
16. American Cancer Society. (2016).
17. D. Hanahan, R. A. Weinberg, The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
18. D. Hanahan, L. M. Coussens, Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* **21**, 309-322 (2012).
19. P. Nyberg, T. Salo, R. Kalluri, Tumor microenvironment and angiogenesis. *Front Biosci* **13**, 6537-6553 (2008).
20. W. E. Mesker *et al.*, The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. *Cell Oncol* **29**, 387-398 (2007).
21. T. J. Dekker *et al.*, Prognostic significance of the tumor-stroma ratio: validation study in node-negative premenopausal breast cancer patients from the EORTC perioperative chemotherapy (POP) trial (10854). *Breast Cancer Res. Treat* **139**, 371-379 (2013).
22. E. M. de Kruijf *et al.*, Tumor-stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. *Breast Cancer Res Treat* **125**, 687-696 (2011).
23. M. Morikawa, R. Derynck, K. Miyazono, TGF-beta and the TGF-beta Family: Context-Dependent Roles in Cell and Tissue Physiology. *Cold Spring Harb Perspect Biol* **8**, (2016).
24. M. Y. Wu, C. S. Hill, Tgf-beta superfamily signaling in embryonic development and homeostasis. *Dev Cell* **16**, 329-343 (2009).
25. K. Kitisin *et al.*, Tgf-Beta signaling in development. *Sci STKE* **2007**, cm1 (2007).
26. Y. Shi, J. Massague, Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700 (2003).
27. R. J. Akhurst, R. W. Padgett, Matters of context guide future research in TGFbeta superfamily signaling. *Sci Signal* **8**, re10 (2015).
28. J. Massague, TGFbeta signalling in context. *Nat. Rev. Mol. Cell Biol*, (2012).
29. R. A. Clark, G. A. McCoy, J. M. Folkvord, J. M. McPherson, TGF-beta 1 stimulates cultured human fibroblasts to proliferate and produce tissue-like fibroplasia: a fibronectin matrix-dependent event. *J Cell Physiol* **170**, 69-80 (1997).

30. A. B. Roberts *et al.*, Type beta transforming growth factor: a bifunctional regulator of cellular growth. *Proc. Natl. Acad. Sci. U. S. A* **82**, 119-123 (1985).
31. S. L. Lu *et al.*, Loss of transforming growth factor-beta type II receptor promotes metastatic head-and-neck squamous cell carcinoma. *Genes Dev* **20**, 1331-1342 (2006).
32. N. M. Munoz *et al.*, Transforming growth factor beta receptor type II inactivation induces the malignant transformation of intestinal neoplasms initiated by Apc mutation. *Cancer Res* **66**, 9837-9844 (2006).
33. J. Wang *et al.*, Targeted disruption of Smad4 in cardiomyocytes results in cardiac hypertrophy and heart failure. *Circ Res* **97**, 821-828 (2005).
34. S. Biswas *et al.*, Transforming growth factor beta receptor type II inactivation promotes the establishment and progression of colon cancer. *Cancer Res* **64**, 4687-4692 (2004).
35. E. Forrester *et al.*, Effect of conditional knockout of the type II TGF-beta receptor gene in mammary epithelia on mammary gland development and polyomavirus middle T antigen induced tumor formation and metastasis. *Cancer Res* **65**, 2296-2302 (2005).
36. R. Derynck, R. J. Akhurst, Differentiation plasticity regulated by TGF-beta family proteins in development and disease. *Nat Cell Biol* **9**, 1000-1004 (2007).
37. J. P. Thiery, Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* **15**, 740-746 (2003).
38. S. A. Mani *et al.*, The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704-715 (2008).
39. R. Kalluri, EMT: when epithelial cells decide to become mesenchymal-like cells. *J Clin Invest* **119**, 1417-1419 (2009).
40. R. Kalluri, R. A. Weinberg, The basics of epithelial-mesenchymal transition. *J Clin Invest* **119**, 1420-1428 (2009).
41. M. T. Jennings, J. A. Pietenpol, The role of transforming growth factor beta in glioma progression. *J Neurooncol* **36**, 123-140 (1998).
42. D. Padua *et al.*, TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* **133**, 66-77 (2008).
43. J. J. Yin *et al.*, TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* **103**, 197-206 (1999).
44. Y. Kang *et al.*, A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* **3**, 537-549 (2003).
45. L. A. Kingsley, P. G. Fournier, J. M. Chirgwin, T. A. Guise, Molecular biology of bone metastasis. *Mol Cancer Ther* **6**, 2609-2617 (2007).
46. Y. Kang *et al.*, Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci U S A* **102**, 13909-13914 (2005).
47. C. Neuzillet *et al.*, Targeting the TGFbeta pathway for cancer therapy. *Pharmacol Ther* **147**, 22-31 (2015).
48. F. Dituri *et al.*, Differential Inhibition of the TGF-beta Signaling Pathway in HCC Cells Using the Small Molecule Inhibitor LY2157299 and the D10 Monoclonal Antibody against TGF-beta Receptor Type II. *PLoS One* **8**, e67109 (2013).
49. J. Dzieran *et al.*, Comparative analysis of TGF-beta/Smad signaling dependent cytostasis in human hepatocellular carcinoma cell lines. *PLoS One* **8**, e72252 (2013).
50. M. Serova *et al.*, Effects of TGF-beta signalling inhibition with galunisertib (LY2157299) in hepatocellular carcinoma models and in ex vivo whole tumor tissue samples from patients. *Oncotarget* **6**, 21614-21627 (2015).
51. C. Neuzillet *et al.*, Perspectives of TGF-beta inhibition in pancreatic and hepatocellular carcinomas. *Oncotarget* **5**, 78-94 (2014).
52. S. Medicherla *et al.*, Antitumor activity of TGF-beta inhibitor is dependent on the microenvironment. *Anticancer Res* **27**, 4149-4157 (2007).
53. A. Mazzocca, E. Fransvea, G. Lavezzari, S. Antonaci, G. Giannelli, Inhibition of transforming growth factor beta receptor I kinase blocks hepatocellular carcinoma growth through neo-angiogenesis regulation. *Hepatology* **50**, 1140-1151 (2009).
54. A. Akbari *et al.*, Evaluation of antitumor activity of a TGF-beta receptor I inhibitor (SD-208) on human colon adenocarcinoma. *Daru* **22**, 47 (2014).

55. J. Zhang, S. Sud, K. Mizutani, M. R. Gyetko, K. J. Pienta, Activation of urokinase plasminogen activator and its receptor axis is essential for macrophage infiltration in a prostate cancer mouse model. *Neoplasia* **13**, 23-30 (2011).
56. M. Zhang *et al.*, Blockade of TGF-beta signaling by the TGFbetaR-I kinase inhibitor LY2109761 enhances radiation response and prolongs survival in glioblastoma. *Cancer Res* **71**, 7155-7167 (2011).
57. M. Uhl *et al.*, SD-208, a novel transforming growth factor beta receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo. *Cancer Res* **64**, 7954-7961 (2004).
58. T. T. Tran *et al.*, Inhibiting TGF-beta signaling restores immune surveillance in the SMA-560 glioma model. *Neuro Oncol* **9**, 259-270 (2007).
59. L. Gil-Guerrero *et al.*, In vitro and in vivo down-regulation of regulatory T cell activity with a peptide inhibitor of TGF-beta1. *J Immunol* **181**, 126-135 (2008).
60. S. Kim *et al.*, Systemic blockade of transforming growth factor-beta signaling augments the efficacy of immunogene therapy. *Cancer Res* **68**, 10247-10256 (2008).
61. D. Llopiz *et al.*, Peptide inhibitors of transforming growth factor-beta enhance the efficacy of antitumor immunotherapy. *Int J Cancer* **125**, 2614-2623 (2009).
62. H. Tanaka *et al.*, Transforming growth factor beta signaling inhibitor, SB-431542, induces maturation of dendritic cells and enhances anti-tumor activity. *Oncol Rep* **24**, 1637-1643 (2010).
63. E. B. Wilson *et al.*, Human tumour immune evasion via TGF-beta blocks NK cell activation but not survival allowing therapeutic restoration of anti-tumour activity. *PLoS One* **6**, e22842 (2011).
64. K. Garrison *et al.*, The small molecule TGF-beta signaling inhibitor SM16 synergizes with agonistic OX40 antibody to suppress established mammary tumors and reduce spontaneous metastasis. *Cancer Immunol Immunother* **61**, 511-521 (2012).
65. J. Park *et al.*, Combination delivery of TGF-beta inhibitor and IL-2 by nanoscale liposomal polymeric gels enhances tumour immunotherapy. *Nat Mater* **11**, 895-905 (2012).
66. S. Oh *et al.*, Transforming growth factor-beta gene silencing using adenovirus expressing TGF-beta1 or TGF-beta2 shRNA. *Cancer Gene Ther* **20**, 94-100 (2013).
67. Z. Xu, Y. Wang, L. Zhang, L. Huang, Nanoparticle-delivered transforming growth factor-beta siRNA enhances vaccination against advanced melanoma by modifying tumor microenvironment. *ACS Nano* **8**, 3636-3645 (2014).
68. B. Zhang, S. K. Halder, S. Zhang, P. K. Datta, Targeting transforming growth factor-beta signaling in liver metastasis of colon cancer. *Cancer Lett* **277**, 114-120 (2009).
69. J. Nemunaitis *et al.*, Phase II study of belagenpumatucel-L, a transforming growth factor beta-2 antisense gene-modified allogeneic tumor cell vaccine in non-small-cell lung cancer. *J Clin Oncol* **24**, 4721-4730 (2006).
70. J. Olivares *et al.*, Phase I trial of TGF-beta 2 antisense GM-CSF gene-modified autologous tumor cell (TAG) vaccine. *Clin Cancer Res* **17**, 183-192 (2011).
71. N. Senzer *et al.*, Phase I trial of "bi-shRNAi(furin)/GMCSF DNA/autologous tumor cell" vaccine (FANG) in advanced cancer. *Mol Ther* **20**, 679-686 (2012).
72. J. C. Morris *et al.*, Phase I study of GC1008 (fresolimumab): a human anti-transforming growth factor-beta (TGFbeta) monoclonal antibody in patients with advanced malignant melanoma or renal cell carcinoma. *PLoS One* **9**, e90353 (2014).
73. J. Folkman, P. Cole, S. Zimmerman, Tumor behavior in isolated perfused organs: in vitro growth and metastases of biopsy material in rabbit thyroid and canine intestinal segment. *Ann. Surg* **164**, 491-502 (1966).
74. P. Carmeliet, R. K. Jain, Angiogenesis in cancer and other diseases. *Nature* **407**, 249-257 (2000).
75. J. Folkman, E. Merler, C. Abernathy, G. Williams, Isolation of a tumor factor responsible for angiogenesis. *J. Exp. Med* **133**, 275-288 (1971).
76. Y. Kang, C. R. Chen, J. Massague, A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Mol Cell* **11**, 915-926 (2003).
77. T. Sanchez-Elsner *et al.*, Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. *J Biol Chem* **276**, 38527-38535 (2001).
78. D. J. Waugh, C. Wilson, The interleukin-8 pathway in cancer. *Clin Cancer Res* **14**, 6735-6741 (2008).

79. A. E. Koch *et al.*, Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* **258**, 1798-1801 (1992).
80. R. M. Strieter *et al.*, Interleukin-8. A corneal factor that induces neovascularization. *Am J Pathol* **141**, 1279-1284 (1992).
81. P. Carmeliet *et al.*, Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* **98**, 147-157 (1999).
82. T. Kawasaki *et al.*, A requirement for neuropilin-1 in embryonic vessel formation. *Development* **126**, 4895-4902 (1999).
83. B. Sitohy, J. A. Nagy, H. F. Dvorak, Anti-VEGF/VEGFR therapy for cancer: reassessing the target. *Cancer Res* **72**, 1909-1914 (2012).
84. E. U. Cidon, P. Alonso, B. Masters, Markers of Response to Antiangiogenic Therapies in Colorectal Cancer: Where Are We Now and What Should Be Next? *Clin Med Insights Oncol* **10**, 41-55 (2016).
85. N. E. Sounni *et al.*, Blocking lipid synthesis overcomes tumor regrowth and metastasis after antiangiogenic therapy withdrawal. *Cell Metab* **20**, 280-294 (2014).
86. G. Bergers, D. Hanahan, Modes of resistance to anti-angiogenic therapy. *Nat. Rev. Cancer* **8**, 592-603 (2008).
87. P. ten Dijke, H. M. Arthur, Extracellular control of TGFbeta signalling in vascular development and disease. *Nat. Rev. Mol. Cell Biol* **8**, 857-869 (2007).
88. E. Perez-Gomez *et al.*, The role of the TGF-beta coreceptor endoglin in cancer. *ScientificWorldJournal* **10**, 2367-2384 (2010).
89. J. Massague, R. R. Gomis, The logic of TGFbeta signaling. *FEBS Lett* **580**, 2811-2820 (2006).
90. M. J. Goumans *et al.*, Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Mol. Cell* **12**, 817-828 (2003).
91. F. Lebrin *et al.*, Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J* **23**, 4018-4028 (2004).
92. D. W. Miller *et al.*, Elevated expression of endoglin, a component of the TGF-beta-receptor complex, correlates with proliferation of tumor endothelial cells. *Int. J. Cancer* **81**, 568-572 (1999).
93. M. J. Goumans *et al.*, Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* **21**, 1743-1753 (2002).
94. M. A. Brown *et al.*, Crystal structure of BMP-9 and functional interactions with pro-region and receptors. *J Biol Chem* **280**, 25111-25118 (2005).
95. M. Scharpfenecker *et al.*, BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *J. Cell Sci* **120**, 964-972 (2007).
96. S. P. Oh *et al.*, Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A* **97**, 2626-2631 (2000).
97. D. Y. Li *et al.*, Defective angiogenesis in mice lacking endoglin. *Science* **284**, 1534-1537 (1999).
98. H. M. Arthur *et al.*, Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev. Biol* **217**, 42-53 (2000).
99. A. Bourdeau, D. J. Dumont, M. Letarte, A murine model of hereditary hemorrhagic telangiectasia. *J. Clin. Invest* **104**, 1343-1351 (1999).
100. F. Lebrin, M. Deckers, P. Bertolino, P. ten Dijke, TGF-beta receptor function in the endothelium. *Cardiovasc. Res* **65**, 599-608 (2005).
101. M. Paauwe, P. ten Dijke, L. J. Hawinkels, Endoglin for tumor imaging and targeted cancer therapy. *Expert. Opin. Ther. Targets* **17**, 421-435 (2013).
102. S. I. Cunha *et al.*, Endothelial ALK1 Is a Therapeutic Target to Block Metastatic Dissemination of Breast Cancer. *Cancer Res* **75**, 2445-2456 (2015).
103. S. I. Cunha *et al.*, Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. *J Exp Med* **207**, 85-100 (2010).
104. A. Necchi *et al.*, PF-03446962, a fully-human monoclonal antibody against transforming growth-factor beta (TGFbeta) receptor ALK1, in pre-treated patients with urothelial cancer: an open label, single-group, phase 2 trial. *Invest New Drugs* **32**, 555-560 (2014).

105. L. A. van Meeteren *et al.*, Anti-human activin receptor-like kinase 1 (ALK1) antibody attenuates bone morphogenetic protein 9 (BMP9)-induced ALK1 signaling and interferes with endothelial cell sprouting. *J Biol Chem* **287**, 18551-18561 (2012).

106. L. S. Rosen *et al.*, A Phase I First-in-Human Study of TRC105 (Anti-Endoglin Antibody) in Patients with Advanced Cancer. *Clin. Cancer Res* **18**, 4820-4829 (2012).

107. C. Anderberg *et al.*, Deficiency for endoglin in tumor vasculature weakens the endothelial barrier to metastatic dissemination. *J. Exp. Med* **210**, 563-579 (2013).

108. O. de Wever, M. Mareel, Role of tissue stroma in cancer cell invasion. *J. Pathol* **200**, 429-447 (2003).

109. D. Ruiter, T. Bogenrieder, D. Elder, M. Herlyn, Melanoma-stroma interactions: structural and functional aspects. *Lancet Oncol* **3**, 35-43 (2002).

110. Y. Crawford *et al.*, PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. *Cancer Cell* **15**, 21-34 (2009).

111. N. Erez, M. Truitt, P. Olson, S. T. Arron, D. Hanahan, Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. *Cancer Cell* **17**, 135-147 (2010).

112. T. Tsujino *et al.*, Stromal myofibroblasts predict disease recurrence for colorectal cancer. *Clin Cancer Res* **13**, 2082-2090 (2007).

113. P. Surowiak *et al.*, Occurence of stromal myofibroblasts in the invasive ductal breast cancer tissue is an unfavourable prognostic factor. *Anticancer Res* **27**, 2917-2924 (2007).

114. H. Sugimoto, T. M. Mundel, M. W. Kieran, R. Kalluri, Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol Ther* **5**, 1640-1646 (2006).

115. C. Anderberg *et al.*, Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts. *Cancer Res* **69**, 369-378 (2009).

116. M. Augsten, Cancer-associated fibroblasts as another polarized cell type of the tumor microenvironment. *Front Oncol* **4**, 62 (2014).

117. J. Tommelein *et al.*, Cancer-associated fibroblasts connect metastasis-promoting communication in colorectal cancer. *Front Oncol* **5**, 63 (2015).

118. L. Ronnov-Jessen, O. W. Petersen, V. E. Koteliansky, M. J. Bissell, The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. *J Clin Invest* **95**, 859-873 (1995).

119. M. P. Lewis *et al.*, Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *Br J Cancer* **90**, 822-832 (2004).

120. S. S. McAllister *et al.*, Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell* **133**, 994-1005 (2008).

121. S. S. McAllister, R. A. Weinberg, Tumor-host interactions: a far-reaching relationship. *J Clin Oncol* **28**, 4022-4028 (2010).

122. N. C. Direkze *et al.*, Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer Res* **64**, 8492-8495 (2004).

123. G. Ishii *et al.*, Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. *Biochem Biophys Res Commun* **309**, 232-240 (2003).

124. M. Quante *et al.*, Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. *Cancer Cell* **19**, 257-272 (2011).

125. M. Elkabets *et al.*, Human tumors instigate granulin-expressing hematopoietic cells that promote malignancy by activating stromal fibroblasts in mice. *J Clin Invest* **121**, 784-799 (2011).

126. E. L. Spaeth *et al.*, Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PLoS One* **4**, e4992 (2009).

127. P. J. Mishra *et al.*, Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. *Cancer Res* **68**, 4331-4339 (2008).

128. A. Schmitt-Graff, A. Desmouliere, G. Gabbiani, Heterogeneity of myofibroblast phenotypic features: an example of fibroblastic cell plasticity. *Virchows Arch* **425**, 3-24 (1994).

129. D. C. Radisky, P. A. Kenny, M. J. Bissell, Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? *J Cell Biochem* **101**, 830-839 (2007).

130. E. M. Zeisberg, S. Potenta, L. Xie, M. Zeisberg, R. Kalluri, Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res* **67**, 10123-10128 (2007).
131. A. Armulik, G. Genove, C. Betsholtz, Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev. Cell* **21**, 193-215 (2011).
132. N. A. Bhowmick *et al.*, TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* **303**, 848-851 (2004).
133. H. Beppu *et al.*, Stromal inactivation of BMPRII leads to colorectal epithelial overgrowth and polyp formation. *Oncogene* **27**, 1063-1070 (2008).
134. M. T. Dimanche-Boitrel *et al.*, In vivo and in vitro invasiveness of a rat colon-cancer cell line maintaining E-cadherin expression: an enhancing role of tumor-associated myofibroblasts. *Int J Cancer* **56**, 512-521 (1994).
135. K. Pietras, A. Ostman, Hallmarks of cancer: interactions with the tumor stroma. *Exp. Cell Res* **316**, 1324-1331 (2010).
136. J. Massague, TGFbeta in Cancer. *Cell* **134**, 215-230 (2008).
137. A. Calon *et al.*, Dependency of Colorectal Cancer on a TGF-beta-Driven Program in Stromal Cells for Metastasis Initiation. *Cancer Cell* **22**, 571-584 (2012).
138. O. De Wever *et al.*, Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. *FASEB J* **18**, 1016-1018 (2004).
139. A. De Boeck *et al.*, Bone marrow-derived mesenchymal stem cells promote colorectal cancer progression through paracrine neuregulin 1/HER3 signalling. *Gut* **62**, 550-560 (2013).
140. M. Van Bockstal *et al.*, Differential regulation of extracellular matrix protein expression in carcinoma-associated fibroblasts by TGF-beta1 regulates cancer cell spreading but not adhesion. *Oncoscience* **1**, 634-648 (2014).
141. W. Lederle *et al.*, MMP13 as a stromal mediator in controlling persistent angiogenesis in skin carcinoma. *Carcinogenesis* **31**, 1175-1184 (2010).
142. C. Gaggioli *et al.*, Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol* **9**, 1392-1400 (2007).
143. D. T. Fearon, The carcinoma-associated fibroblast expressing fibroblast activation protein and escape from immune surveillance. *Cancer Immunol. Res* **2**, 187-193 (2014).
144. J. Zhang, L. Chen, M. Xiao, C. Wang, Z. Qin, FSP1+ fibroblasts promote skin carcinogenesis by maintaining MCP-1-mediated macrophage infiltration and chronic inflammation. *Am J Pathol* **178**, 382-390 (2011).
145. M. Kraman *et al.*, Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science* **330**, 827-830 (2010).
146. J. Harper, R. C. Sainson, Regulation of the anti-tumour immune response by cancer-associated fibroblasts. *Semin. Cancer Biol* **25**, 69-77 (2014).
147. F. Balkwill, K. A. Charles, A. Mantovani, Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* **7**, 211-217 (2005).
148. G. Comito *et al.*, Cancer-associated fibroblasts and M2-polarized macrophages synergize during prostate carcinoma progression. *Oncogene* **33**, 2423-2431 (2014).
149. H. Salmon, E. Donnadieu, Within tumors, interactions between T cells and tumor cells are impeded by the extracellular matrix. *Oncoimmunology* **1**, 992-994 (2012).
150. A. M. Scott *et al.*, A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer. *Clin Cancer Res* **9**, 1639-1647 (2003).
151. K. Narra *et al.*, Phase II trial of single agent Val-boroPro (Talabostat) inhibiting Fibroblast Activation Protein in patients with metastatic colorectal cancer. *Cancer Biol Ther* **6**, 1691-1699 (2007).
152. E. P, Uber den jetzigen Stand der Karzinomforschung. *Nederlands Tijdschrift voor Geneeskunde* **5**, 273-290 (1909).
153. M. W. Teng, J. B. Swann, C. M. Koebel, R. D. Schreiber, M. J. Smyth, Immune-mediated dormancy: an equilibrium with cancer. *J Leukoc Biol* **84**, 988-993 (2008).
154. R. Kim, M. Emi, K. Tanabe, Cancer immunoediting from immune surveillance to immune escape. *Immunology* **121**, 1-14 (2007).
155. F. Pages *et al.*, Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* **29**, 1093-1102 (2010).

156. S. Ostrand-Rosenberg, Immune surveillance: a balance between protumor and antitumor immunity. *Curr Opin Genet Dev* **18**, 11-18 (2008).

157. M. Karin, T. Lawrence, V. Nizet, Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* **124**, 823-835 (2006).

158. K. E. de Visser, A. Eichten, L. M. Coussens, Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* **6**, 24-37 (2006).

159. H. F. Dvorak, Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* **315**, 1650-1659 (1986).

160. M. Schafer, S. Werner, Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Biol* **9**, 628-638 (2008).

161. S. I. Grivennikov, F. R. Greten, M. Karin, Immunity, inflammation, and cancer. *Cell* **140**, 883-899 (2010).

162. L. Yang, Y. Pang, H. L. Moses, TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends Immunol* **31**, 220-227 (2010).

163. J. D. Shields, I. C. Kourtis, A. A. Tomei, J. M. Roberts, M. A. Swartz, Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science* **328**, 749-752 (2010).

164. D. Mougiakakos, A. Choudhury, A. Lladser, R. Kiessling, C. C. Johansson, Regulatory T cells in cancer. *Adv Cancer Res* **107**, 57-117 (2010).

165. J. Sheng, W. Chen, H. J. Zhu, The immune suppressive function of transforming growth factor-beta (TGF-beta) in human diseases. *Growth Factors* **33**, 92-101 (2015).

166. D. G. DeNardo, P. Andreu, L. M. Coussens, Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev* **29**, 309-316 (2010).

167. J. A. Joyce, D. T. Fearon, T cell exclusion, immune privilege, and the tumor microenvironment. *Science* **348**, 74-80 (2015).

168. F. Balkwill, Cancer and the chemokine network. *Nat Rev Cancer* **4**, 540-550 (2004).

169. B. Bottazzi *et al.*, Chemotactic activity for mononuclear phagocytes of culture supernatants from murine and human tumor cells: evidence for a role in the regulation of the macrophage content of neoplastic tissues. *Int J Cancer* **31**, 55-63 (1983).

170. A. Mantovani *et al.*, Chemokines in the recruitment and shaping of the leukocyte infiltrate of tumors. *Semin Cancer Biol* **14**, 155-160 (2004).

171. J. R. Reed *et al.*, Fibroblast growth factor receptor 1 activation in mammary tumor cells promotes macrophage recruitment in a CX3CL1-dependent manner. *PLoS One* **7**, e45877 (2012).

172. T. Ueno *et al.*, Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res* **6**, 3282-3289 (2000).

173. S. K. Biswas, A. Mantovani, Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* **11**, 889-896 (2010).

174. A. Mantovani, S. Sozzani, M. Locati, P. Allavena, A. Sica, Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* **23**, 549-555 (2002).

175. C. Murdoch, A. Giannoudis, C. E. Lewis, Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood* **104**, 2224-2234 (2004).

176. S. M. Zeisberger *et al.*, Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. *Br J Cancer* **95**, 272-281 (2006).

177. V. Gocheva *et al.*, IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev* **24**, 241-255 (2010).

178. R. Wang *et al.*, Tumor-associated macrophages provide a suitable microenvironment for non-small lung cancer invasion and progression. *Lung Cancer* **74**, 188-196 (2011).

179. J. Y. Lin, X. Y. Li, N. Tadashi, P. Dong, Clinical significance of tumor-associated macrophage infiltration in supraglottic laryngeal carcinoma. *Chin J Cancer* **30**, 280-286 (2011).

180. W. Qing *et al.*, Density of tumor-associated macrophages correlates with lymph node metastasis in papillary thyroid carcinoma. *Thyroid* **22**, 905-910 (2012).

181. O. Vasiljeva *et al.*, Tumor cell-derived and macrophage-derived cathepsin B promotes progression and lung metastasis of mammary cancer. *Cancer Res* **66**, 5242-5250 (2006).

182. B. Qian *et al.*, A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PLoS One* **4**, e6562 (2009).
183. N. Gungor *et al.*, Genotoxic effects of neutrophils and hypochlorous acid. *Mutagenesis* **25**, 149-154 (2010).
184. J. K. Sandhu, H. F. Privora, G. Wenckebach, H. C. Birnboim, Neutrophils, nitric oxide synthase, and mutations in the mutatect murine tumor model. *Am J Pathol* **156**, 509-518 (2000).
185. J. E. De Larco, B. R. Wuertz, L. T. Furcht, The potential role of neutrophils in promoting the metastatic phenotype of tumors releasing interleukin-8. *Clin Cancer Res* **10**, 4895-4900 (2004).
186. P. Scapini *et al.*, CXCL1/macrophage inflammatory protein-2-induced angiogenesis in vivo is mediated by neutrophil-derived vascular endothelial growth factor-A. *J Immunol* **172**, 5034-5040 (2004).
187. J. Jablonska, S. Leschner, K. Westphal, S. Lienenklaus, S. Weiss, Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model. *J Clin Invest* **120**, 1151-1164 (2010).
188. M. M. Queen, R. E. Ryan, R. G. Holzer, C. R. Keller-Peck, C. L. Jorcyk, Breast cancer cells stimulate neutrophils to produce oncostatin M: potential implications for tumor progression. *Cancer Res* **65**, 8896-8904 (2005).
189. S. J. Huh, S. Liang, A. Sharma, C. Dong, G. P. Robertson, Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. *Cancer Res* **70**, 6071-6082 (2010).
190. Z. Granot *et al.*, Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* **20**, 300-314 (2011).
191. Z. G. Fridlender *et al.*, Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. *Cancer Cell* **16**, 183-194 (2009).
192. K. Shang *et al.*, Crucial involvement of tumor-associated neutrophils in the regulation of chronic colitis-associated carcinogenesis in mice. *PLoS One* **7**, e51848 (2012).
193. T. Jamieson *et al.*, Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis. *J Clin Invest* **122**, 3127-3144 (2012).
194. M. Quintanilla *et al.*, Expression of the TGF-beta coreceptor endoglin in epidermal keratinocytes and its dual role in multistage mouse skin carcinogenesis. *Oncogene* **22**, 5976-5985 (2003).
195. E. Perez-Gomez *et al.*, A role for endoglin as a suppressor of malignancy during mouse skin carcinogenesis. *Cancer Res* **67**, 10268-10277 (2007).
196. L. A. Henry *et al.*, Endoglin expression in breast tumor cells suppresses invasion and metastasis and correlates with improved clinical outcome. *Oncogene* **30**, 1046-1058 (2011).
197. V. C. Wong *et al.*, Identification of an invasion and tumor-suppressing gene, Endoglin (ENG), silenced by both epigenetic inactivation and allelic loss in esophageal squamous cell carcinoma. *Int. J. Cancer* **123**, 2816-2823 (2008).
198. E. Pardali *et al.*, Critical role of endoglin in tumor cell plasticity of Ewing sarcoma and melanoma. *Oncogene* **30**, 334-345 (2011).
199. D. Romero *et al.*, Endoglin regulates cancer-stromal cell interactions in prostate tumors. *Cancer Res* **71**, 3482-3493 (2011).
200. A. Orlidge, P. A. D'Amore, Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol* **105**, 1455-1462 (1987).
201. K. B. Saunders, P. A. D'Amore, An in vitro model for cell-cell interactions. *In Vitro Cell Dev Biol* **28A**, 521-528 (1992).
202. Y. Kubota, H. K. Kleinman, G. R. Martin, T. J. Lawley, Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol* **107**, 1589-1598 (1988).
203. A. M. Goodwin, In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. *Microvasc Res* **74**, 172-183 (2007).
204. I. Arnaoutova, J. George, H. K. Kleinman, G. Benton, The endothelial cell tube formation assay on basement membrane turns 20: state of the science and the art. *Angiogenesis* **12**, 267-274 (2009).
205. K. L. DeCicco-Skinner *et al.*, Endothelial cell tube formation assay for the in vitro study of angiogenesis. *J Vis Exp*, e51312 (2014).
206. F. Pampaloni, E. G. Reynaud, E. H. Stelzer, The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol* **8**, 839-845 (2007).

207. T. Korff, H. G. Augustin, Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J Cell Biol* **143**, 1341-1352 (1998).

208. T. Korff, S. Kimmina, G. Martiny-Baron, H. G. Augustin, Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J* **15**, 447-457 (2001).

209. S. Boyden, The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J Exp Med* **115**, 453-466 (1962).

210. N. P. Jobe *et al.*, Simultaneous blocking of IL-6 and IL-8 is sufficient to fully inhibit CAF-induced human melanoma cell invasiveness. *Histochem Cell Biol*, (2016).

211. L. Bochet *et al.*, Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer. *Cancer Res* **73**, 5657-5668 (2013).

212. D. Herrmann *et al.*, Three-dimensional cancer models mimic cell-matrix interactions in the tumour microenvironment. *Carcinogenesis* **35**, 1671-1679 (2014).

213. S. Zhao, J. Huang, J. Ye, A fresh look at zebrafish from the perspective of cancer research. *J Exp Clin Cancer Res* **34**, 80 (2015).

214. A. V. Gore, K. Monzo, Y. R. Cha, W. Pan, B. M. Weinstein, Vascular development in the zebrafish. *Cold Spring Harb Perspect Med* **2**, a006684 (2012).

215. J. Kanungo, E. Cuevas, S. F. Ali, M. G. Paule, Zebrafish model in drug safety assessment. *Curr Pharm Des* **20**, 5416-5429 (2014).

216. A. V. Kalueff, A. M. Stewart, R. Gerlai, Zebrafish as an emerging model for studying complex brain disorders. *Trends Pharmacol Sci* **35**, 63-75 (2014).

217. J. R. Guyon *et al.*, Modeling human muscle disease in zebrafish. *Biochim Biophys Acta* **1772**, 205-215 (2007).

218. B. Weinstein, Vascular cell biology in vivo: a new piscine paradigm? *Trends Cell Biol* **12**, 439-445 (2002).

219. G. J. Lieschke, A. C. Oates, M. O. Crowhurst, A. C. Ward, J. E. Layton, Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood* **98**, 3087-3096 (2001).

220. C. Santoriello, L. I. Zon, Hooked! Modeling human disease in zebrafish. *J Clin Invest* **122**, 2337-2343 (2012).

221. R. M. White *et al.*, Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* **2**, 183-189 (2008).

222. C. Tobia, G. De Sena, M. Presta, Zebrafish embryo, a tool to study tumor angiogenesis. *Int J Dev Biol* **55**, 505-509 (2011).

223. S. Nicoli, D. Ribatti, F. Cotelli, M. Presta, Mammalian tumor xenografts induce neovascularization in zebrafish embryos. *Cancer Res* **67**, 2927-2931 (2007).

224. I. J. Marques *et al.*, Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model. *BMC Cancer* **9**, 128 (2009).

225. S. He *et al.*, Neutrophil-mediated experimental metastasis is enhanced by VEGFR inhibition in a zebrafish xenograft model. *J Pathol* **227**, 431-445 (2012).

226. J. Jonkers, A. Berns, Conditional mouse models of sporadic cancer. *Nat Rev Cancer* **2**, 251-265 (2002).

227. O. M. Rashid, K. Takabe, Animal models for exploring the pharmacokinetics of breast cancer therapies. *Expert Opin Drug Metab Toxicol* **11**, 221-230 (2015).

228. P. W. Derksen *et al.*, Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell* **10**, 437-449 (2006).

229. M. De Robertis *et al.*, The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. *J Carcinog* **10**, 9 (2011).

230. T. Tanaka *et al.*, A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* **94**, 965-973 (2003).

231. C. Neufert, C. Becker, M. F. Neurath, An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression. *Nat. Protoc* **2**, 1998-2004 (2007).