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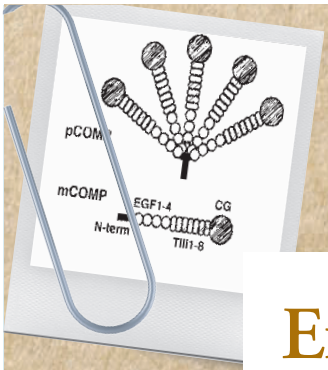

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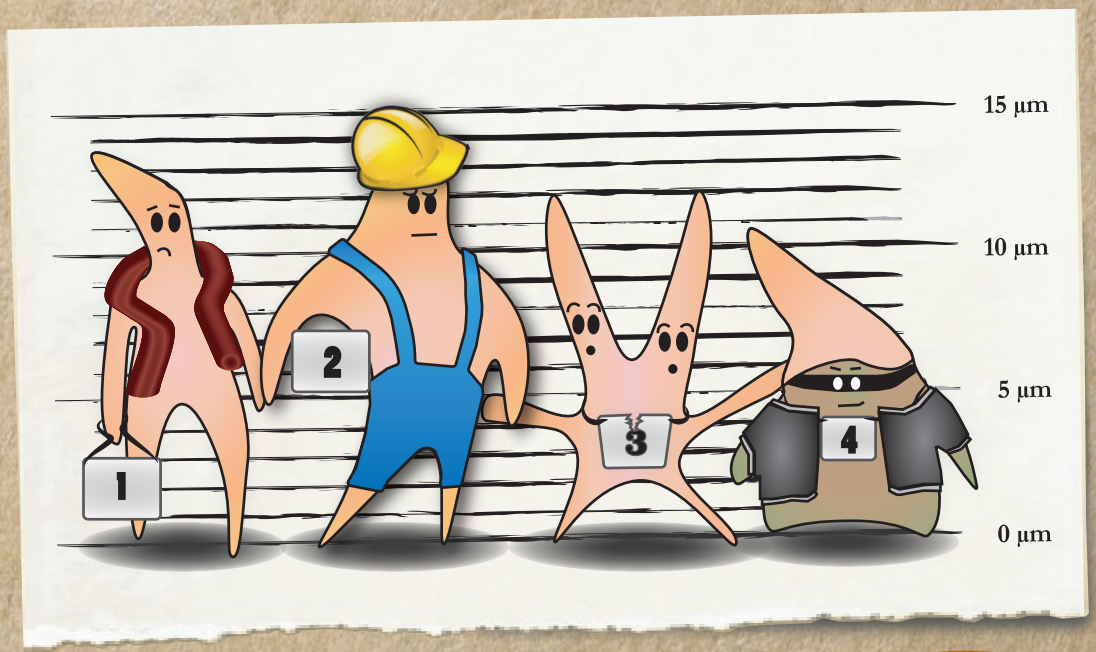

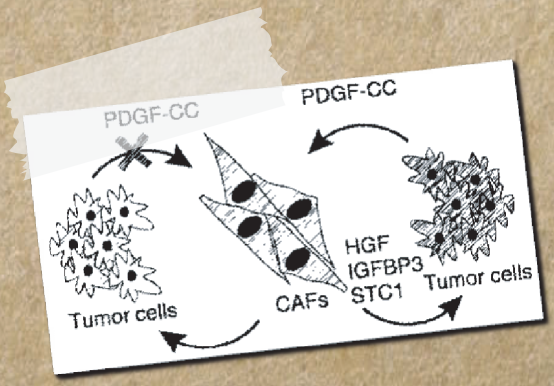

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Abstract

The tumor microenvironment consists of several interacting cell types. Cancer research focssed mainly on the malignant cell in the past. The importance of the tumor microenvironment is increasingly appreciated, as endothelial cells and immune cells were identified as targets for anti-tumor therapy. Targeted therapy against cancer-associated fibroblasts (CAFs) are not in clinical use for the treatment of carcinomas, even though CAFs are involved in many tumor-supporting processes. CAFs are mesenchymal stromal cells and generate and modulate the extracellular matrix (ECM), which provides physical stability to the growing tumor. CAFs can alter cell-to-cell communication within the tumor microenvironment and thereby influence the immune reaction to cancer cells, the response to cancer therapy and the tumor metabolism.

Breast cancer is the most common malignant disease and second most common reason for cancer-related death in women. Despite advancements in the treatment of breast cancer, some aggressive forms remain hard to treat.

In the first paper we investigated the effect of complement oligomeric matrix protein (COMP) on breast cancer. Epithelial COMP expression is associated with reduced survival in breast cancer patients.We showed that COMP resolves endoplasmic reticulum stress and deregulates the cell metabolism, causing increased growth and metastasis *in vivo*. We propose *COMP* expression as a potential prognostic marker in breast cancer.

In the second part of the thesis we analyzed the importance of platelet-derived growth factor (PDGF) signaling in solid tumors in general, and the effect of PDGF-CC signaling in breast cancer in particular. We showed that PDGF-CC signaling to CAFs and the subsequent release of CAF-derived stanniocalcin 1, hepatocyte growth factor, and insulin growth factor binding protein 3 maintain a basal-like phenotype in breast cancer. Genetic and pharmacologic disruption of this commuication loop resulted in conversion of a hormone receptor-negative into a hormone receptor-positive state, causing enhanced sensitivity to endocrine therapy in previously resistant tumors. We conclude that the breast cancer subtype is in part under the control of the tumor microenvironment.

CAFs have many different functions in the tumor microenvironment and different origins for CAFs have been suggested. In the last paper we used single-cell RNA-sequencing of 786 mesenchymal cells derived from tumors of the MMTV-PyMT mouse model of breast cancer, to identify subclasses of CAFs in an unbiased approach. We detected and confirmed the existence of four subclasses that potentially derive from three different origins. Based on differential gene expression analysis we assigned functional properties to each CAF subgroup. Gene profiles of the main CAF subgroups held independent prognostic capability in large clinical cohorts. We showed that an in depth investigation of cellular constituents of the tumor microenvironment with increased resolution, can reveal a higher order of cellular organization in malignant disease.

Key words: Cancer-associated fibroblasts, breast cancer, single-cell RNA-sequencing, PDGF

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MADE IN SWEDEN

Wir können alles schaffen, genau wie die toll´n
dressierten Affen wir müssen nur woll´n.

Judith Holofernes

Content

List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Cartilage oligomeric matrix protein contributes to the development and metastasis of breast cancer. Englund E, **Bartoschek M**, Reitsma B, Jacobsson L, Escudero-Esparza A, Orimo A, Leandersson K, Hagerling C, Aspberg A, Storm P, Okroj M, Mulder H, Jirström K, Pietras K, Blom AM, Oncogene, 27.10.2016.

II. PDGF family function and prognostic value in tumor biology. **Bartoschek M** & Pietras K. Biochemical and Biophysical Research Communications, 22.06.2018

III. Microenvironmental control of breast cancer subtype elicited through paracrine platelet derived growth factor-CC signaling. Roswall P*, Bocci M*, **Bartoschek M*,** Li H*, Kristiansen G, Jansson S, Lehn S, Sjölund J, Reid S, Larsson C, Eriksson P, Anderberg C, Cortez E, Saal LH, Ostmark-Pietras C, Cordero E, Haller BK, Häkkinen J, Burvenich IJG, Lim E, Orimo A, Höglund M, Rydén L, Moch H, Scott AM, Eriksson U, Pietras K, Nature Medicine, 12.03.2018, * indicates equal contribution.

IV. Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell RNA sequencing. **Bartoschek M**, Oskolkov N, Bocci M, Lövrot J, Larsson C, Sommarin M, Lindgren D, Karlsson G, Rignér M, Bergh J, Björklund Å, Pietras K, manuscript under revision

Reprints were made with the permission of the respective journal.

Paper not included in the thesis

Complement inhibitor CSMD1 acts as tumor suppressor in human breast cancer. Astrid Escudero-Esparza, **Michael Bartoschek***, Chrysostomi Gialeli*, Marcin Okroj, Sioned Owen, Karin Jirström, Akira Orimo, Wen G. Jiang, Kristian Pietras, Anna M. Blom, Oncotarget 2016, * indicates equal contribution.

List of abbreviations

ALK1	Activin-receptor-like kinase 1
αSMA	Alpha smooth muscle actin
CAF	Cancer-associated fibroblast
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CLS	Crown-like structures
COMP	Cartilage oligomeric matrix protein
CXCL	CXC-motive chemokine ligand
C5AR2/GPR77	Complement component 5a receptor 2
DCN	Decorin
ECM	Extracellular matrix
EGF	Epithelial growth factor
EMT	Epithelial to mesenchymal transition
EndMT	Endothelial to mesenchymal transition
ER	Estrogen receptor
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ERS	Endoplasmic reticulum stress
FACS	Fluorescence activated cell sorting
FAP	Fibroblast activation protein alpha
FGF	Fibroblast growth factor
FSP1	Fibroblast specific protein 1
GBM	Glioblastoma multiforme
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
IFP	Interstitial fluid pressure
IGF	Insulin-like growth factor
IGFBP3	IGF binding protein 3
IL	Interleukin
LOX	Lysyl oxidase

MDSC	Myeloid-derived suppressor cell
MME	Membrane metalloendopeptidase
MMP	Matrix metallopeptidase
MMTV	Mouse mammary tumor virus
NHG	Nottingham histological grade
NK	Natural killer
NOD	Non-obese diabetic
NSG	NOD-SCID-gamma
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PDX	Patient-derived xenograft
PFT	Pericyte to fibroblast transition
PR	Progesterone receptor
PyMT	Polyoma middle T antigen
RGS5	Regulator of G protein signaling 5
SCID	Severe combined immunodeficiency
Shh	Sonic hedgehog
SMART	Switching mechanism at 5’ end of RNA template
STC1	Stanniocalcin 1
TGFβ	Transforming growth factor beta
TIII	Thrombospondin type 3
TIMP	Tissue inhibitors of metalloproteinases
tPA	Tissue plasminogen activator
Treg	Regulatory T cell
UMI	Unique molecular identifiers
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor

Introduction

Tumors are complex entities consisting of several interacting cell types (figure 1). Initially, the efforts of cancer research focused on the malignant cancer cells within tumors. Malignant cells are capable to escape the tight regulation of cell division displayed by their healthy counterparts mainly through acquisition of mutations. However, these malignant cells are not able to form solid tumors, or even hematologic neoplasms, without the support of specialized cells of the tumor microenvironment. The supply of nutrients and oxygen to the tumor depends on blood vessels made up of endothelial cells and pericytes. Fibroblasts generate and modulate the extracellular matrix (ECM), which provides physical stability to the growing tumor and can alter cell-to-cell communication. The role of immune cells within the tumor microenvironment is ambiguous: they can either act against the tumor growth by killing malignant cells, or can support the malignant progression of a tumor by maintaining an inflammatory environment. Malignant cells can corrupt regulatory immune cells to tame an immune response and generate immune tolerance of the developing tumor. Cancer cells exploit the complex network of cell-to-cell signaling to evoke these tumor-supportive reactions of the local microenvironment.

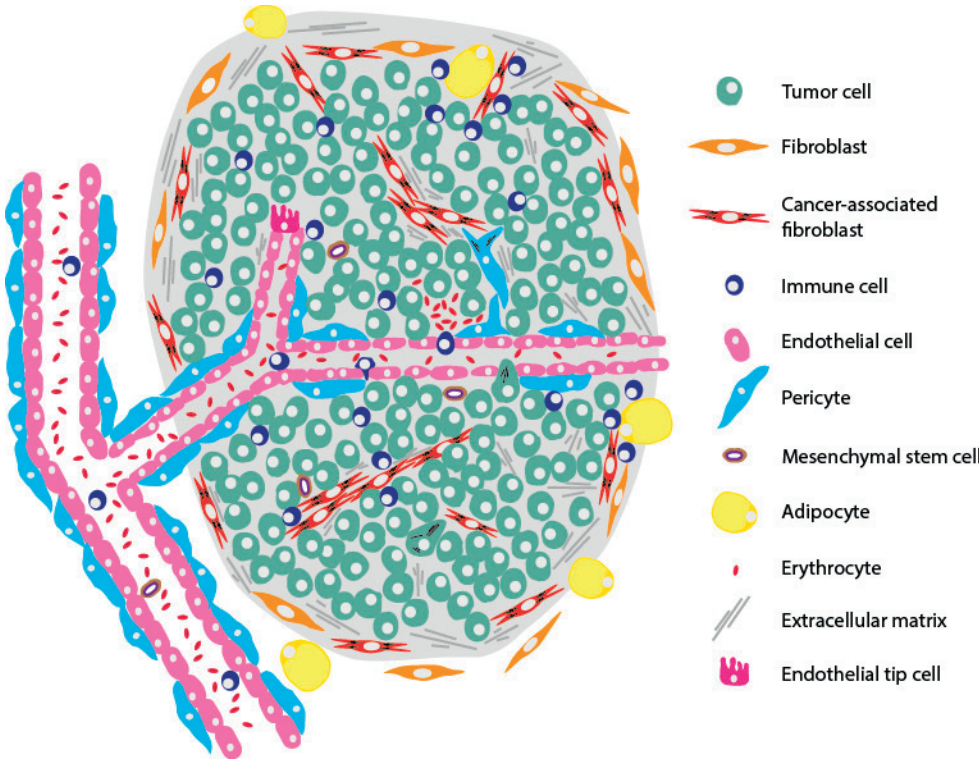

Figure 1: Schematic representation of the tumor microenvironment
A solid tumor consists of malignant epithelial cells, cancer-associated fibroblasts, endothelial cells, pericytes and the extracellular matrix. Immune cells and mesenchymal stem cells invade the tumor through the blood stream. Adipocytes are found in the surrounding breast tissue. Endothelial tip cells lead the direction of a sprouting vessel.

In the following sections I will introduce the main cell types of the tumor microenvironment followed by a brief summary of breast carcinoma, as it is the main disease this thesis deals with. I will continue with the most important proteins for the included publications, the platelet-derived growth factor (PDGF) family of proteins and cartilage oligomeric matrix protein (COMP), as well as an introduction of the most important methodologies.

Malignant cells

Cancer cells arise from healthy cells that escaped the tight regulation of cell proliferation. The increased cell proliferation leads to the formation and expansion of a primary tumor, ultimately superseding the healthy tissue at the place of origin. While this basic progress is common to all tumors, the diseases referred to a cancer are a highly heterogeneous group of individual diseases.

Based on the tissue of origin, tumors are classified into carcinomas (epithelial), sarcomas (mesenchymal), leukemias and lymphomas (hematopoietic). Within these categories, different subclasses exist and add to the complexity and heterogeneity of the disease.

The heterogeneity becomes apparent when studying the numerous ways through which malignant cells gain the ability to proliferate indefinitely, evade growth suppression by the surrounding tissue or overcome programmed cell death. Despite the differences on the molecular and histological level, the standard treatment for many different kinds of cancers targets their common functional characteristic, the increased proliferation rate: in addition to surgical removal of the tumor growth, radiation- and chemotherapy are administered to interfere with ordered cell division and thus proliferation.

Decades of research attempting to impinge on the so-called hallmarks of cancer (1,2) led to the development of more specific and targeted cancer therapies. In addition to the heterogeneity through different tissues of origin, the same cancer types can manifest differently in different patients and even within a single tumor cancer cells can be highly diverse in their genotype and phenotype. These differences highlight the importance of individualized treatments. Moreover, the intra-tumor heterogeneity further complicates the response to treatment, as the discriminating pressure of drugs might select for resistant or more aggressive clones (3).

When a primary tumor was removed and a patient was treated with chemotherapy, malignant cells might have survived in a dormant state. Cancer dormancy is either characterized by a mitotic growth arrest (cellular dormancy), or the growth arrest of a cancer mass, that remains in a state of equilibrium between proliferation and apoptosis (4). The equilibrium can be sustained by the lack of oxygen and nutrients in the microenvironment of the dormant tumor mass, or the control of the immune system preventing an outgrowth. The malignant cell mass can survive for several years in a dormant state and lead to recurrence when a potential stimulus tips the balance. Since the treatment and processes during dormancy selected for the most robust cells, recurrences tend to be more aggressive than the initial tumor.

Depending on the location of growth, the time of detection and the treatment, a primary tumor can cause organ failure at the site of its occurrence. For instance, tumors within the peritoneum might not be palpable in early stages and can progress without apparent symptoms, causing detection at late and therefore fatal stages. Furthermore, tumors can cause systemic syndromes like cachexia. Cachexia is a metabolic syndrome characterized by loss of skeletal muscle and in some cases adipose tissue and immunological competence (5). It has been proposed that cachexia is driven by inflammation. The physiological response of a systemic inflammation is substrate mobilization through catabolic degradation of tissues, in order to cover the demand of molecular building blocks required for the developing immune response. In the case of cancer, this substrate mobilization can also fuel tumor growth.

According to the literature, despite the adverse effects of primary tumor growth, the major cause of death in cancer is the development of metastatic lesions in distant organs (6). The process of metastasis is still not fully understood, but is known to require cancer cells to detach from the primary site, travel as single cells or cell clusters through the bloodstream or lymph vessels and finally invade a tissue at a distant location. During this process, cancer cells are exposed to entirely different environments as well as physical stress.

Depending on the tumor type, some organs are preferred destinations for metastatic spread. Stephen Paget's "seed and soil" hypothesis states, that disseminating tumor cells (seed) need preferable interactions with the organ-specific microenvironment (soil) in order to progress to a metastatic lesion (7,8). Recent studies suggest, that a primary tumor is able to prepare the "soil" from the distance, for instance through systemically active signaling molecules or exosomes (9).

It has been suggested that the process of epithelial-mesenchymal transition (EMT) plays a role in metastasis of carcinomas, more specifically in the process of cell detachment from the primary site of the tumor (10). EMT is a key process during development. For example, it is required for gastrulation and implantation of the embryo, as cells of epithelial origin gain mesenchymal features like motility and lose epithelial features like tissue connectivity. Moreover, EMT plays a role in wound healing (11). The process of EMT has been described as transient, dynamic and reversible with a continuous spectrum between the epithelial and mesenchymal state (12,13). Developmental biologists and cancer biologists identified several genes that are expressed during EMT. The transcription factors Snail, Slug, Twist and Zeb1 are the most commonly used to identify EMT. The main function of these transcription factors is the repression of epithelial genes like E-cadherin (14). Tumor cells can supposedly hijack this process in order to escape the primary tumor and metastasize.

The hypothesis of EMT being involved in metastasis has been challenged by Zheng and colleagues. They showed that the knock-out of EMT regulators *SNAI1* and *TWIST1* was not rate-limiting for invasion and metastasis in a mouse model of pancreatic ductal adenocarcinoma (PDAC) (15). Based on these observations, they propose that EMT is not a primary requirement for metastasis. The lineage tracing study in lung adenocarcinoma by Fischer and colleagues also supports the theory that metastatic processes are independent of EMT (16). However, the marker fibroblast specific protein 1 (FSP1), which was used for EMT lineage tracing in this study, might not be suitable, as mesenchymal cells express varying sets of marker genes, which will be discussed in the section "Mesenchymal cells".

Despite these results, EMT should not be completely excluded as a mechanism involved in metastasis formation. EMT is a highly complex process involving several transcription factors and genes. From a detection point of view, only following a single gene to trace lineage transitions might not suffice to track the process. Biologically, the knock-out of only one transcription factor at a time might not stop the process entirely. A partial EMT is potentially still sufficient for tumor cells to metastasize (17).

Immune cells

The main function of immune cells is to protect an organism against pathogens without reacting to itself and allowing symbionts to survive where needed. This balance of activation and repression is a highly complex system of pattern recognition receptors and signaling pathways. Immune cells are mobile and can, with some exceptions like brain and testis (18), enter any tissue in order to detect and kill pathogens or infected cells. The theory of immunoediting states that there are three distinct stages of interaction between the immune system and cancer cells: elimination, equilibrium and escape (19). During elimination, the immune system is able to overcome its tolerance to itself and can detect and remove most cells that underwent malignization. Distinguishing malignant from non-malignant cells is possible due to the aberrant gene expression and the occurrence of tumor specific antigens in malignant cells. If elimination of malignant cells cannot be completed, the phase of equilibrium marks a transition where the tumor is held at bay by the immune system. During the equilibrium phase cancer cells can develop further mechanisms to avoid immune recognition and eventually the system enters the escape phase. At this stage, tumor cells that were able to resist, avoid, or suppress the antitumor immune response can proliferate further, resulting in tumor growth that cannot be contained by the immune system any longer.

Immunotherapy aims to target the mechanisms that malignant cells develop in order to evade destruction by the immune system and allow them to transition into the escape phase. The most successful recent approach is to block the immuno-suppressive signaling between programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and their respective ligands PD-L1/2 and CD80/CD86 with so-called immune checkpoint inhibitors (20-22). PD1-and CTLA-4 are expressed on T-cells to downregulate the immune response, prohibiting an over-reaction of the immune system (23). In pathological conditions, malignant cells can start expressing their ligands in order to tame activated immune cells (24). The CTLA-4 inhibitor ipilimumab has been approved by the FDA for treatment of advanced melanoma, colorectal cancer and renal cell carcinoma, and is currently tested in clinical trials for treatment of several other cancer types, such as advanced hormone-refractory prostate cancer, bladder cancer, non-small cell lung carcinoma and small cell lung cancer (25). Similarly, PD-1 inhibitors nivolumab and pembrolizumab are approved for melanoma, lung cancer and lymphoma, with many ongoing clinical trials in various cancer types. Atezolizumab targets PD-L1 and was approved for treatment of colorectal cancer, melanoma, breast cancer and renal cell carcinoma. The field of immunotherapy emphasizes how targeting the tumor microenvironment can lead to new therapeutic approaches.

Endothelial cells

Endothelial cells are highly organized cells forming tubes that make up the inner lining of blood and lymphatic vessels. Blood vessels are found in all organs, as they are necessary to transport nutrients and oxygen to tissues. In early stages of development, endothelial cells are generated from precursors originating from the mesodermal germ layer in a process called vasculogenesis. In later stages of development and in adulthood, new blood vessels form from pre-existing ones in a process called angiogenesis. The following summary aims to introduce some key players of angiogenesis.

Low oxygen saturation in tissues induces the activation of hypoxia-inducible factor (HIF)-1α which itself induces the expression of vascular endothelial growth factor A (VEGFA), fibroblast growth factor (FGF), PDGF, angiopoietin and SD1α (26). In sprouting angiogenesis, VEGFA signaling through VEGF receptor 2 (VEGFR2) leads to the activation of endothelial cells (27,28). Activated endothelial cells loosen endothelial junctions and become invasive. At each branching point one endothelial cell becomes a tip cell which leads the way of the growing vessel. The tip cell is supported and followed by proliferating stalk cells. The proliferation of stalk cells is orchestrated by the function of Notch and Wnt

signaling preventing the formation of further tip cells (29,30). Gradients of VEGF and other growth factors sequestered in the ECM are sensed by tip cells and direct the way of the growing vessel. Among these other growth factors are FGFs, PDGF, Angiopoietins and transforming growth factor β (TGFβ).

FGF and VEGFA signaling synergize during angiogenesis. FGF-1 and FGF-2 drive proliferation and differentiation in angiogenesis and vessel maturation and lead to the upregulation of VEGFA and VEGFRs.

Endothelial cell- and tip cell-derived PDGF-BB attracts pericytes to associate with immature vessels and to orchestrate vessel stabilization (31,32).

Angiopoietins (Ang1 and Ang2) signal through the receptor Tie2 on endothelial cells. Ang1 mediates endothelial cell survival, proliferation, migration and anti-inflammatory signals. Ang2 inhibits Tie2 signaling in the presence of Ang1 and moderately activates Tie2 in the absence of Ang1 (33). Endothelial cells produce and release Ang2 during activation as an autocrine regulator of Ang/Tie2 signaling (34).

The TGFβ superfamily of ligands and receptors is important in vessel development. Endothelial cells almost exclusively express the TGFβ receptor activin-receptor-like kinase 1 (ALK1) and endoglin. Knock-outs of the genes *Acvrl1* and *Eng* encoding ALK1 and endoglin, respectively, are embryonically lethal in mice, because of failed vascularization of the yolk sac and the lack of smooth muscle cell differentiation (35). Hereditary hemorrhagic telangiectasia, a vascular disease characterized by abnormal blood vessels and vessel lesions is linked to mutations in *Acvrl1* and *Eng*.

Based on observations made by Algire, Chalkley, Greenblatt and Shubik (36-38), Folkman proposed that solid tumors induce angiogenesis in order to gain access to nutrients and oxygen, which are both necessary for proliferation (36). The transition of a tumor between a non-angiogenic to an angiogenic state is called the angiogenic switch. It was proposed that the ratio of stimulators and inhibitors of angiogenesis determines whether angiogenesis is blocked or activated (39). In contrast to tissue development, tumor-induced angiogenesis is not as tightly regulated, since tumor cells produce angiogenic growth factors in an uncontrolled fashion. They also induce further growth factor secretion in fibroblasts and immune cells through maintenance of an inflammatory environment. In addition to sprouting angiogenesis, further mechanisms of tumor vascularization, such as vessel co-option, vascular mimicry, intussusception, postnatal vasculogenesis and endothelial differentiation of cancer stem cells have been identified (40). Vascularization does not only facilitate supply of oxygen and nutrients but also provides cancer cells with an escape route from the tumor and allows for dissemination as metastases.

The initial goal of anti-angiogenic therapy was to reduce the amount of blood vessels and thereby cutting the supply of oxygen and nutrients through the bloodstream. Despite promising preclinical results of VEGF neutralizing antibodies such as bevacizumab, and VEGF traps like aflibercept, VEGF–neutralizing anti-angiogenic drugs performed below expectation in the clinic. Specifically, in breast cancer, the use of bevacizumab did not increase overall survival, as a short response phase of delayed growth is followed by an adaptation phase and the development of resistances (41).

The persistent VEGF signaling within tumors inhibits vessel maturation, causing leakiness and disorganization of the vascular tree (42). An alternative therapeutic strategy involving vascularization is the modulation of the VEGF-signaling rather than completely blocking angiogenesis through VEGF-inhibitors. Modulating angiogenic signaling could lead to vessel normalization, which has been suggested to be beneficial as functional vessels enable increased drug delivery (43,44). The majority of signaling pathways in angiogenesis are based on tyrosine kinase receptors. Small molecule tyrosine kinase inhibitors like sunitinib, sorafenib and axitinib target several receptor tyrosine kinases to various extents and ranges of specificity and are already approved for treatment of several tumor types. Sunitinib for example is used for the treatment of gastrointestinal stromal tumors (GIST), pancreatic cancer and renal cell carcinoma (25).

Mesenchymal cells

The mesenchyme is a form of connective tissue found in embryogenesis. It derives from the mesoderm and contains mesenchymal stem cells that give rise to mesenchymal cells including osteoblasts, chondrocytes, myocytes, adipocytes, pericytes and fibroblasts. In carcinomas, therapies specifically targeting mesenchymal cells in the tumor microenvironment are not in clinical use, even though small molecule receptor tyrosine kinase inhibitors bear target specificity against receptors mainly found on mesenchymal cells. In contrast to this, in soft tissue sarcomas which originate from mesenchymal cells, the PDGF receptor α (PDGFRα) targeting antibody olaratumab is used in combination with doxorubicin (45).

Pericytes

Pericytes are a type of perivascular mural cells. Endothelial cells encompassed by perivascular mural cells form a vascular unit. In the microvasculature, the mural cells are commonly pericytes, whereas arterioles and venules are invested by

vascular smooth muscle cells (46). Key characteristics of pericytes are their contractility, the embedding into the basement membrane and protruding extensions that they use to maintain contact to endothelial cells (47). Pericyte coverage of endothelial cells is associated to vascular impermeability and therefore found to be increased in brain vessels compared to other tissues. In agreement with this, a regulatory role of pericytes in blood flow and vessel stability was suggested (48).

Pericytes also produce the constituents of the basement membrane, which they share with endothelial cells within the vascular unit (49). Furthermore, pericytes were reported to display a high degree of plasticity as they can differentiate into other cell types such as adipocytes, chondrocytes, osteoblasts and skeletal muscle. They are able to dedifferentiate to cells with mesenchymal stem cell properties (50-53).

Identification of pericytes

Pericytes are hard to define by their expressed molecular markers. Suggested marker genes display variable expression patterns between pericytes derived from different organs and are not expressed exclusively on pericytes (54). Therefore, the most reliable way to detect pericytes is still by morphology and spatial association to endothelial cells. Most common pericyte markers for histological detection used in the literature are sulfate proteoglycan 4 (CSPG4 or NG2) (55), PDGFRβ (31), α-smooth muscle actin (αSMA) (56), regulator of G protein signaling 5 (RGS5) (57) and desmin (58).

Pericyte-endothelial cell interaction

In general, pericytes are attracted to growing vessels by PDGF-BB secreted by immature endothelial cells and endothelial tip cells (31,32). In addition, there is PDGF-BB independent recruitment of pericytes to the growing vasculature, for instance in the development of liver vessels and vessels of the central nervous system (32).

The main function of pericytes in their interaction with endothelial cells is to stabilize vessels and to promote endothelial survival by inducing autocrine VEGF signaling in endothelial cells (49,59). On the other hand, pericytes in a proangiogenic environment can themselves induce endothelial cell proliferation and sprouting by secreting VEGF-A (60). Pericytes further regulate late processes in sprouting angiogenesis by selectively inducing vessel regression, which has been shown in both pathological and physiological conditions (61).

In tumor vasculature, pericytes are less tightly attached to endothelial cells and cytoplasmic processes can stretch into the tumor tissue instead of keeping the contact to endothelial cells (62). In several tumor types pericyte coverage is

reduced compared to the respective healthy tissue (62,63). Nevertheless, pericytes are essential for functional tumor vasculature and are therefore potential targets in combination with anti-angiogenic therapy targeting endothelial cells.

Combinations of VEGFR (endothelial cells) and PDGFR (pericytes) targeting drugs semaxanib (SU5416) and Gleevec (Imatinib), reduced tumor size even of late stage tumors in a mouse model of pancreatic neuroendocrine cancer (RIP1-Tag2) (64). In the same mouse model, dual targeting of PDGFR and VEGFR in combination with a "chemo-switch" protocol, involving sequential maximum tolerated dose and metronomic treatment with cyclophosphamide, resulted in complete responses or lesions of reduced size (65). These results, combined with the tight interaction of the tumor microenvironment, suggest that further combination treatment targeting mixed cell types could improve cancer therapy.

Adipocytes

Adipocytes are specialized in energy storage through the accumulation of lipids in large unilocular droplets in their cytoplasm. However, they are not only passive storage cells, as adipocytes can regulate the metabolism and energy turnover in an endocrine-dependent fashion through the secretion of adipokines (66). Adipocyte precursor cells called preadipocytes have fibroblast morphology and high proliferative activity (67).

Adipose tissue has been linked to increased inflammation, based on the presence of the crown-like structures (CLS), consisting of dying adipocytes surrounded by macrophages. CLS have been linked to proinflammatory processes (68). Although they are also found in normal adipose tissue, they are increased in individuals with higher body mass index and in postmenopausal breast tissue. It was even suggested that CLS play a role in tumor initiation (69).

Cancer adjacent adipocytes display a distinctly changed transcriptional profile compared to distant adipocytes (70). Adipocytes can promote tumor growth by providing energy (71), and by the secretion of adipokines and growth factors such as leptin, adiponectin, IL-6, TNF-α and hepatocyte growth factor (HGF) (72). Adipocytes were suggested to dedifferentiate into CAFs, in this way contributing to invasion and metastasis (73,74).

Fibroblasts

Fibroblasts were first characterized by their shape and position in tissues. Fibroblasts are motile, contractile and, depending on their activation status, they appear as spindle-shaped or stellate-shaped cells. This morphology and their lack of specific marker expression for other cell lineages are predominantly used to

identify fibroblasts. Fibroblasts are mainly found in connective tissue where they are the main producers of the ECM (75). Both fibrillary collagen types I, II, III and V, as well as the basement membrane components collagen type IV and laminin, are secreted by fibroblasts (76-78). ECM modification through crosslinking and degradation is facilitated by the action of enzymes also secreted by fibroblasts (77,79-81). Examples of these enzymes are matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and lysil oxidases (LOX).

Fibroblasts are receptive to signals from the surrounding tissue and can change their behavior accordingly. For example, upon injury they are activated and migrate to the wound where they produce the scaffold for other cell types to facilitate wound closure (76). Mediators of fibroblast activation are TGF-β, chemokines such as monocyte chemotactic protein 1, ECM-degrading proteases, degraded ECM fragments, FGF, Wnt7a and the complement protein 5Ca (82-86).

Scar tissue and tissue fibrosis is a result of fibroblast activation and excessive deposition of ECM constituents. In culture, fibroblasts, derived from wounds and fibrotic tissue, secrete increased amounts of ECM compared to resident fibroblasts from other tissues (87).

Fibroblasts not only receive signals, but also produce signaling factors to communicate with other cell types. These factors can influence epithelial cell differentiation and regulation of the immune response (76,88). Fibroblast-secreted proteolytic enzymes can set free signaling molecules bound to the ECM through ECM degradation (89).

The activation status and tissue of origin largely affects the gene expression of fibroblasts (90). The rigidity of the fibroblast environment influences gene expression. Standardized conditions such as tissue culture medium and growth on hard plastic surfaces might level the variance of fibroblast functions observed *in vivo*. It is therefore imperative to reduce the time in culture to investigate fibroblast heterogeneity.

Fibroblasts in cancer

In many solid tumors, fibroblasts make up the majority of the non-malignant stroma and are called tumor-associated fibroblasts, carcinoma-associated fibroblasts, reactive stromal fibroblasts or cancer-associated fibroblasts (CAFs) (91). Since tumors are often described as wounds that do not heal, CAFs are also referred to as activated fibroblasts (92). Early studies showed that cancer-associated fibroblasts, in contrast to normal tissue fibroblasts, can support the growth of malignant epithelium, suggesting that normal tissue fibroblasts in proximity of a lesion become educated to support tumorigenesis (93).

In the initial phases of tumorigenesis, the malignant cells are still confined by the basal lamina and thereby separated from the surrounding tissue. At this stage they are called carcinoma *in situ*. It is not fully understood how a carcinoma *in situ* progresses to an invasive state, but there is evidence for a supportive role of CAFs in this process (94). CAFs directly support tumor growth by secreting growth factors such as HGF, epithelial growth factor (EGF), insulin like growth factor 1 (IGF-1) and C-X-C motif chemokine ligand 12 (CXCL12) also known as stromal cell-derived factor-1. Furthermore, CAFs indirectly stimulate malignant progression by secreting proangiogenic VEGF, fibroblast growth factor 2 (FGF-2), interleukin-8 (IL-8) and platelet-derived growth factor C (PDGF-C) (95-97). CAF-derived CXCL14 promotes tumor growth and attracts immune cells but also stimulates growth and migration of CAFs themselves (98).

Tumor cells can change their glucose metabolism from oxidative phosphorylation, requiring oxygen, to anaerobe glycolysis, which is known as the Warburg effect (99). It has been reported that CAFs are also able to adjust their metabolism to engage in a metabolic symbiosis with the tumor cells (100). In this context, a switch to an anaerobic metabolism in CAFs leaves more oxygen to the tumor cells and provides metabolites that serve as building blocks for proliferating cells (101).

CAFs are able to protect tumor cells from the immune system by attracting immune-suppressive regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) to the tumor (102). Moreover, CAFs suppress cytotoxic $CD8^+$ T cells through a mechanism dependent on immune checkpoint activation (103). In contrast to these immune-suppressive functions, CAFs can fuel constant inflammation in the tumor microenvironment by attracting macrophages (88,104). Physiological inflammation, for instance during wound healing, is characterized by signaling cascades stimulating immune cell proliferation and the regeneration of the injured tissue (105). Tumor cells are able to exploit these signaling cues to promote growth, motility and invasion. Additionally, inflammatory cells release highly reactive nitrogen and oxygen species, which can induce further mutations in cancer cells.

CAFs are able to regulate the interstitial fluid pressure (IFP). IFP in the tumor microenvironment is strongly increased compared to normal tissues, eventually resulting in reduced concentrations of systemically administered drugs (106,107). Studies in rodent models showed that treatment with PDGFR targeting drugs reduced IFP and improved the drug uptake in tumors and thereby the efficacy of systemic treatment (108-110). In the investigated model systems, CAFs expressed the targeted PDGF receptors exclusively, suggesting a modulatory function of fibroblasts in IFP. Indeed, CAFs are anchored to the ECM through integrins, conveying physical pressure when contracting, which results in an increased IFP (111).

Besides the direct physical interaction of CAFs with the ECM, CAFs modulate crosslinking of collagen fibers and collagen bundles through the production and secretion of enzymes. Increased crosslinking leads to increased stiffness, which has been linked to aggressiveness and poor survival in breast cancer (112). Nevertheless, the tumor ECM also constitutes a barrier that tumor cells need to overcome in order to invade the surrounding tissue and finally metastasize. CAFs enable tumor cells to escape by proteolytically digesting the ECM, allowing malignant cells to pass through the stromal barrier in the initial steps of the metastatic cascade (113,114). The two seemingly opposing functions, crosslinking and proteolytic degradation, in CAFs, are of importance at different stages of tumorigenesis, suggesting that targeting of CAFs has multiple outcomes that have to be considered during the design of potential targeted treatment regimens.

Not only the escape of tumor cells from the primary tumor can be induced by CAFs: CAF-released signaling molecules were reported to prime the metastatic niche (soil), enabling tumor cells to grow in distant organs (9,115). Therefore, CAFs are involved in many steps of metastasis.

One challenge of cancer treatment in the clinic is the development of resistance to therapy, especially to targeted therapy. Stromal cells, and particularly CAFs can induce resistance through paracrine signaling in the tumor microenvironment. CAF-derived HGF induces RAF-inhibitor resistance in *BRAF*-mutant melanoma, colorectal and glioblastoma cell lines and resistance to selective tyrosine kinase inhibitors to EGFR in lung cancer cells (116,117). CAF-derived nitric oxide induces IL-1β secretion in pancreatic ductal carcinoma cells which is associated with chemoresistance (118).

Origin of CAFs

The origin of CAFs in the tumor mass is still a matter of debate, as there is evidence for several potential sources (figure 2).

While malignant cells expand within a healthy tissue, the first fibroblasts in contact with a nascent tumor will be the resident tissue-specific population. Tumor cell-derived signals can activate resident fibroblasts to become reactive, similar to activation during wound healing (119).

Another potential origin of CAFs is the recruitment of mesenchymal stem cells, which can differentiate into fibroblasts (120).

Epithelial cells or endothelial cells can undergo EMT or a specialized process of endothelial-to-mesenchymal transition (EndMT), respectively (121,122). These processes can give rise to CAFs in a tumor.

CAFs can potentially derive from pericytes. For instance, in kidney fibrosis, pericytes were shown to be a source of fibroblasts (123). Whether CAFs could

originate from pericytes in tumors was long debated (124), but recent work suggests that PDGF-BB-driven pericyte to fibroblast transition (PFT) contributes to the stroma (125).

It is not entirely clear if the origin of CAFs has implications on the functional properties of CAF populations. Conceivably, CAFs derived from tumor cells undergoing EMT would contain mutations accumulated during malignant progression, and therefore potentially display aberrant behavior (126).

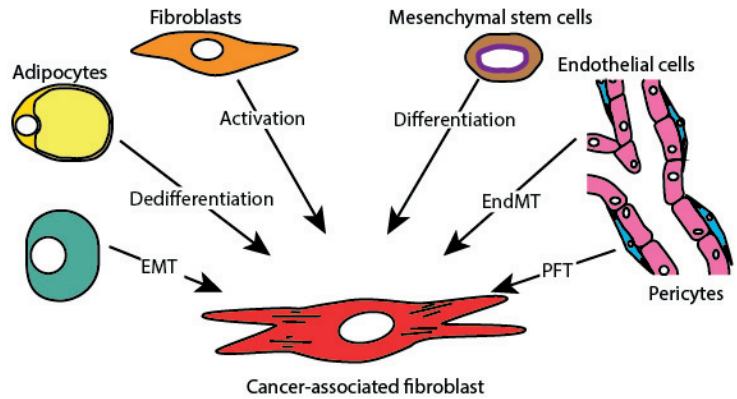

Figure 2: Origins of CAFs
CAFs can originate from resident stromal fibroblasts or adipocytes through activation or dedifferentiation. Mesenchymal stem cells can be recruited to the tumor and differentiate to CAFs. Malignant epithelial cells, endothelial cells and pericytes can undergo epithelial to mesenchymal transition (EMT), endothelial to mesenchymal transition (EndMT) or pericyte to fibroblast transition (PFT) to give rise to CAFs.

CAF markers and subpopulations

Similar to pericytes, the detection of CAFs is not trivial, as there is no known, universal marker gene that is homogeneously expressed by all CAFs (127). Most commonly, CAFs are identified by expression of αSMA, PDGFRα, PDGFRβ , FSP-1/S100A4 and/or fibroblast activation protein (FAP). However, the expression of the listed marker proteins is not exclusive for CAFs or fibroblasts in general. Furthermore, CAFs can express different sets of these marker genes at variable levels, complicating their detection and characterization in the tumor microenvironment.

Given the potential origins and broad spectrum of functions, it has been suggested that CAFs consist of functional subclasses, like it is known for myeloid and lymphoid cells (127). Several subclasses of CAFs have been postulated recently (Table 1).

Öhlund and colleagues identified two CAF subclasses in murine pancreatic ductal adenocarcinoma, based on three marker genes and their localization. FAP^{+} αSMA^{high}, myofibroblastic CAFs were found in direct proximity to neoplastic cells

whereas αSMA^{low} IL6^{high} inflammatory CAFs with a secretory phenotype were mainly found distant to neoplastic cells. Both cell types are plastic and can transform to one another based on the type of interaction with the tumor cells (128).

Li and colleagues used single cell transcriptomics on colorectal cancer samples and identified normal fibroblasts together with two CAF subpopulations: one group was of myofibroblastic origin and expressed *ACTA2*, *TAGLN* and *PDGFA*, whereas the second one was characterized by *MMP2*, *DCN* and *COL1A2* expression (129).

Su and colleagues described a subpopulation of CD10^{+}GPR77^{+} (MME^{+} C5AR2^{+}) fibroblasts through immune staining of patient tissues. Analysis of the patient cohorts revealed that these CAFs correlated with poor prognosis by increasing chemoresistance and by sustaining cancer stemness (130).

Costa and colleagues used a fluorescence-activated cell sorting (FACS) panel of six fibroblast genes to detect four different CAF populations in human breast cancer samples (131). The detected myofibroblastic CAF populations were characterized by either oxidative metabolism or immunosuppression. The different CAF populations were found in different proportions dependent on the molecular subtype of breast cancer. The choice of CAF markers in this study was based on what is known in the literature and included FAP, CD29, PDGFRβ, αSMA, caveolin 1 and FSP1. The initial subdivision on CAF populations was based on the staining for FAP and CD29. The other markers are of minor importance as their expression does not differ strongly between the identified populations.

Table 1: CAF populations in the literature.

Markers	Characteristics	Tumor type	Publicaiton
FAP^{+}, αSMA^{high}	Myofibroblastic, periglandular	mPDAC	Öhlund et al. (128)
IL6^{high}, αSMA ^{low}	Inflammatory, secretory, distant to tumor cells inside the stroma		
αSMA^{+}, TAGLN^{+}, PDGFRA^{+}	Myofibroblasts	hCRC	Li et al. (129)
MMP2^{+}, DCN^{+}, COL1A2^{+}	Extracellular matrix remodeling		
CD10^{+}, GPR77^{+}	Promote tumor formation and chemoresistance, provide niche for cancer stem cells	hBRCA, hLUC	Su et al. (130)
CD29^{med}, FAP^{high}, αSMA^{high}, PDGFRβ$^{Med-Hi*}$	Myofibroblasts, immunosuppression, through T cell attraction and Treg maturation	hBRCA	Costa et al. (131)
CD29^{low}, FAP^{neg}, αSMA^{neg}, PDGFRβ^{neg*}	Stromal cell		
CD29^{med}, FAP^{neg}, αSMA$^{neg-low}$, PDGFRβ^{med*}	Juxta-tumor location		
CD29^{high}, FAP^{neg}, αSMA^{high}, PDGFRβ$^{low-med*}$	Myofibroblasts, oxidative metabolism, actin cytoskeleton		

* indicates shortened list of marker genes. m: murine; h: human. PDAC: pancreatic adenocarcinoma; BRCA: breast Cancer; CRC: colorectal cancer; LUC: lung cancer.

Breast Cancer

Breast cancer is the most common cancer type diagnosed in women (30% in Sweden) and the second most common cause of cancer-related death in women (132). Mortality of breast cancer decreases in industrialized countries, due to improved detection and treatment. Incidences, however, increase constantly in an aging society, as age remains one of the main risk factors (132,133). Breast cancer is not exclusive to women, but is much less common in men (132).

Like most cancer types, breast cancer is a heterogeneous disease. There are several ways to classify breast cancer. TNM classification is based on the clinical stage of breast cancer and takes tumor size and invasiveness (T), lymph node involvement (N) and distant metastasis (M) into account (134). The Nottingham histological grade (NHG) classification is based on histological scores of tubule formation, nuclear atypia and mitotic count and is a measure for the degree of differentiation of the malignant cells (135,136). Poorly or undifferentiated tumors are more aggressive than differentiated tumors.

The majority of breast cancers express estrogen receptor (ER) and/or progesterone receptor (PR). The expression of hormone receptors is a prognostic factor in breast cancer and also predictive for endocrine therapy (137). Endocrine therapy will be explained in the section “Breast cancer treatment”.

Human epidermal growth factor receptor 2 (HER2) is a receptor tyrosine kinase involved in cell adhesion, proliferation, differentiation and cell survival (138). Breast cancer with an amplification of the HER2 encoding gene *ERBB2* and a high histological score is classified as HER2-positive. The proliferation marker Ki67 is used to assess the proliferation rate of breast cancer, adding prognostic value in some cases (139). The division into molecular subtypes of breast cancer will be described in the following section.

Molecular subtypes of breast cancer

In addition to the clinical and histological parameters gene expression data can be used to classify breast cancer into molecular subtypes. These molecular subtypes are luminal A, luminal B, HER2-like, basal-like, normal-like and claudin-low (140,141). In practice, the surrogate markers ER, PR, Ki-67 and HER2 are used to classify the molecular subtypes, following the so called St. Gallen guidelines, which will also determine the treatment regimen and the prognosis (142).

The most common molecular subtypes are luminal A and B accounting for around 70% of all mammary carcinomas. Luminal tumors commonly express ER and/or PR (143). While luminal A tumors are HER2 negative, Ki-67^{low} and have the best

prognosis, luminal B tumors are either HER2 positive or negative with high levels of Ki-67 and a slightly worse prognosis.

HER2-enriched breast cancer is hormone-receptor negative and is characterized by high levels of HER2 expression and amplification of its genetic locus. The prognosis of HER2-enriched tumors is worse compared to the luminal subtype, even though the targeted therapy against HER2 has been introduced in the clinic.

Basal-like tumors account for 10-15 % of breast cancer cases. 70-80 % of basal-like tumors lack the expression of ER, PR and HER2, in which case they are referred to as triple-negative tumors. Out of all breast carcinomas, basal-like tumors display the highest recurrence rate, the shortest time to recurrence and the worst overall survival (143).

Normal-like breast cancer is a rare subtype with expression patterns close to normal breast tissue and fibroadenomas (144). The existence of normal-like tumors has been questioned, as normal breast tissue might have contaminated samples.

Claudin-low tumors are genetically unstable, display low differentiation and are triple-negative in 50% of cases (145). Because there is no clinical indication to categorize claudin-low tumors as a subgroup, they are classified as basal-like tumors (146).

Breast cancer treatment

The first line of treatment for primary breast tumors is dependent on the tumor characteristics, but in most cases is represented by breast-conserving surgery, if possible, followed by radiation therapy (147). The first sentinel lymph nodes, which receive the lymphatic drainage from the primary tumor, are usually removed during primary tumor resection. They are used to evaluate the metastatic dissemination through the lymphatics. After primary tumor resection, radiotherapy eradicates the remaining tumor cells at the site of resection and decreases the risk of local recurrences (148).

Chemotherapy is based on cytotoxic drugs targeting proliferating cells. In breast cancer, chemotherapy is recommended for patients with triple-negative and HER2 positive tumors but also in presence of tumors with high proliferation rate, high histological grade or metastatic spread (149).

Adjuvant endocrine treatment is used to treat ER positive breast cancers, which are detected in the majority (~85%) of patients in Sweden (150). Following international guidelines, a tumor is regarded ER-positive if more than 1% of the malignant epithelium is positive for ER in a standardized staining protocol using immunohistochemistry (149). Endocrine therapy includes selective estrogen

receptor modulators (*e.g.* tamoxifen), selective estrogen receptor degraders (*e.g.* fulvestrant) and aromatase inhibitors, which block estrogen synthesis. According to the St. Gallen guidelines, premenopausal women should be treated with tamoxifen for five years, whereas postmenopausal patients should receive either tamoxifen or aromatase inhibitors for five years (149). Clinical studies suggest that prolonged anti-endocrine treatment for ten years, instead of five, has a preferable outcome for the patients (151).

HER2-enriched breast cancer is treated with trastuzumab, in addition to chemotherapy. In case of low-grade tumors with good prognosis, HER2 targeting treatment is spared (149). Trastuzumab is an antibody targeting the extracellular component of HER2. Its binding inhibits the dimerization of HER2, blocking intracellular signaling cascades (152). In neoadjuvant settings it is recommended to additionally treat with pertuzumab, another HER2 targeting antibody, for a short time period (149).

The therapy of triple-negative breast cancer is still based on surgery, radiation and chemotherapy as targeted therapies are still under investigation.

The use of neoadjuvant chemotherapy to treat breast cancer has been increasing in the past years, based on several rationales. Neoadjuvant chemotherapy can shrink the size of an inoperable tumor to operable size, or allows a more conservative surgery. Patients at risk of distant disease might benefit from preoperative chemotherapy. And lastly, neoadjuvant chemotherapy enables to assess the pathological response to the chosen treatment, which is not possible after surgical removal of the main tumor mass. The pathological response is a valuable prognostic factor to determine overall survival and gives information about the choice of adjuvant therapy. During this “window of opportunity”, further genetic testing and molecular profiling *ex vivo*, enables the choice of a suitable treatment regimen (153).

Despite these potential benefits, critics caution to overuse neoadjuvant chemotherapy based on data from clinical trials (154). The main criticism is that patients are treated even though upfront breast conservation is possible, and that observed responses in primary tumors do not necessarily replicate at metastatic sites. Based on the benefits and criticism it is imperative to improve the selection of patients eligible for neoadjuvant therapy.

PDGF signaling

The PDGF family of ligands and receptors consists of four polypeptide chains that can combine to dimeric ligand isoforms PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD, as well as the two receptor chains PDGFRα and PDGFRβ that can homodimerize and heterodimerize upon activation (figure 3). The following section will introduce the PDGF family in general and the PDGF-CC-PDGFRα signaling pair in detail.

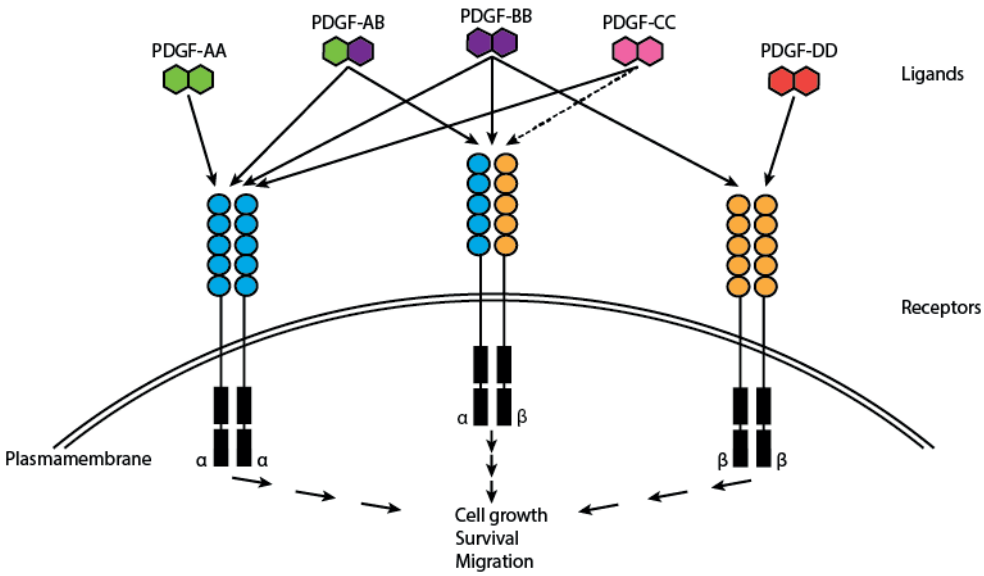

Figure 3: Schematic representation of platelet-derived growth factor (PDGF) signaling
PDGF ligands combine as dimeric isoforms -AA, -AB, -BB, -CC and -DD. Each ligand dimer has specific binding affinity to the dimerized receptor chains. Ligand binding triggers signaling cascades promoting cell growth, survival and migration.

Ligands

The PDGF ligands contain a PDGF/VEGF homology domain which is a conserved growth factor domain of 100 amino acids. As the name indicates, it is shared with members of the VEGF family (155). The growth factor domain alone is sufficient for binding, dimerization and activation of the receptor chains. PDGF-A exists in two variants due to alternative splicing of its mRNA (156). PDGF-B and the longer splice variant of PDGF-A contain a C-terminal retention motive, which enables them to bind to ECM components and to form concentration gradients (156,157). PDGF-AA and BB are secreted as active forms after intracellular activation (158). PDGF-CC and DD, on the other hand, are secreted

as latent forms that require extracellular proteolytic cleavage of an N-terminal CUB domain. Tissue plasminogen activator (tPA) and plasmin have been reported to be involved in the activation of PDGF-CC (159,160). *In vivo,* PDGFs are expressed during development by epithelial (PDGF-A) and endothelial (PDGF-B) cells communicating with mesenchymal cells in close proximity to coordinate tissue and vessel formation (161).

Receptors

The PDGF receptors belong to the type III tyrosine kinase receptor family. PDGF receptors α and β are two structurally related protein chains with five extracellular immunoglobulin-like domains and a split cytoplasmic tyrosine kinase domain (162). Ligand binding causes dimerization followed by cross-phosphorylation of the intracellular domains. In turn, this enables secondary signaling molecules to bind and initiate signaling cascades promoting cell growth, survival and migration *in vitro* (163). Activation of PDGF receptors upon ligand binding is followed by internalization and either degradation in lysosomes or recycling in intracellular vesicles. The five ligand dimers display different binding affinities to the receptor pairs as shown in figure 3.

In breast cancer, PDGFRα is mainly expressed by stromal cells, and rarely by malignant cells (164). High PDGFRα expression in malignant cells is associated with lymph node metastasis, HER2-positivity (165), high histologic grade and hormone receptor negativity (166). High stromal expression of PDGFRα is associated with HER2-positivity and high Ki-67 positivity (164).

Genetic changes in the PDGFR encoding genes can be found in different malignant diseases. Translocations of these genes can generate constitutively active fusion proteins, which were detected in myeloid neoplasms with neutrophilia (167,168). Gain of function point mutations in *PDGFRα* were detected in several different cancer types including glioma, GIST and myeloma (169-171). Some forms of glioblastoma are characterized by amplifications of *PDGFRα* and its overexpression (172).

PDGFR signaling can be blocked by olaratumab, or small molecule receptor tyrosine kinase inhibitors such as imatinib, sunitinib, sorafenib and axitinib, which interfere with the ATP binding to the kinase domain. All these small molecule inhibitors are not specific for PDGFRα, but can inhibit several receptor tyrosine kinases. Imatinib is used to block PDGFR signaling for treatment of the rare tumor types GIST and dermatofibrosarcoma protuberans (173).

PDGF-CC

PDGF-CC was discovered in 2000 and is therefore a relatively new member of the PDGF ligand family (160). PDGF-CC binding specificity was shown for PDGFRα homodimers as well as PDGFRα/β heterodimers *in vitro (160,174,175)*. During brain development, PDGF-CC is required for the formation of the meningeal basement membrane (176). In adult mice, PDGF-CC was found to be expressed in kidney, testis, liver, heart and brain (177). Depending on the genetic background of the mouse model, *Pdgfc* knock-out leads to perinatal lethality, due to a cleft of the secondary palate (178). *Pdgfc*$^{-/-}$ 129S1 and the majority of *Pdgfc*$^{-/-}$ FVB/N mice are affected by this phenotype whereas *Pdgfc*$^{-/-}$ C57BL/6 mice survive until adulthood displaying a milder phenotype (179).

PDGF-CC works mainly as a mitogen in fibroblasts. Macrophage-derived PDGF-CC activates dermal fibroblasts *in vitro*, mimicking wound healing programs after injury (180). In murine models, overexpression of PDGF-CC in the heart causes fibrosis, cardiac hypertrophy and dilated cardiomyopathy (181), whereas in the liver it causes steatosis, fibrosis and the development of hepatocellular carcinoma (182). The effects on the liver are caused by hepatic stellate cells. Upon PDGF-CC signaling, they transform to myofibroblast-like cells and induce chronic inflammation (183).

PDGF-CC and PDGF-AA signaling display almost identical reactions in renal fibroblasts (184). Therefore, PDGF-AA can potentially compensate for the loss of PDGF-CC. PDGF-CC itself can compensate for the loss of VEGFA signaling in the microenvironment of tumors that are resistant to anti-angiogenic therapy (185). The combination of PDGF-CC- and VEGFA-neutralization could potentially improve anti-angiogenic therapy in general, or specifically in tumors that developed resistance to VEGFA-neutralization.

Cartilage oligomeric matrix protein

Cartilage oligomeric matrix protein (COMP) is a pentameric 524 kDa protein that is predominantly expressed in the cartilage (186). Each of its five identical monomers contains four EGF domains, eight thrombospondin type 3 (TIII) domains and a globular C-terminus (187). The monomers are N-terminally linked through a coiled-coil structure and disulfide-bonds. COMP is a calcium-binding glycoprotein and each monomer was predicted to bind ten Ca^{2+} ions, which are important for correct folding and the function of the protein (188).

Mutations in the calcium-binding domain are responsible for the autosomal dominant skeletal disease pseudochondroplasia (189,190). In this disease,

misfolded COMP accumulates in the endoplasmic reticulum of chondrocytes and decreases their viability.

COMP is soluble and facilitates collagen fibrillogenesis in the ECM (191). The main producers of COMP are cells of mesenchymal origin such as dermal fibroblasts (192) and chondrocytes (186) in both healthy and pathological conditions.

COMP plays a role in several diseases. COMP expression is associated with fibrotic conditions in scleroderma (193), which refers to a group of diseases affecting the connective tissue triggered by auto-immune reactions (194). COMP is indeed involved in innate immunity and inflammation (195). COMP activates the alternative complement pathway leading to deposition of C3b and C9. Serum levels of COMP were found to be increased in rheumatoid arthritis and osteoarthritis due to the increased cartilage turnover (196,197).

Even though COMP was investigated mainly in the field of fibrosis, it's role in cancer is increasingly addressed in the literature. COMP expression was associated to unfavorable outcome in colorectal adenocarcinoma (198), prostate cancer (199) and breast cancer (Paper I).

Model systems of cancer

Modeling of cancer in a laboratory environment is essential for preclinical research. Tissue culture and more sophisticated *in vitro* systems, such as tumor spheres can be used for drug screening and the analysis of a drug's molecular mechanism of action. Mouse models are fundamental tools for preclinical research as they fill the gap between the extremely controlled environment and simplicity of tissue culture and the complexity and variability of clinical research. Most mouse models in preclinical research are based on mouse strains in which all mice are genetically identical. These strains were obtained by several rounds of backcrossing. The use of genetically identical mice simplifies research, as genetic variance does not influence the experimental results. The following section will deal with general concepts in mouse models of cancer and the cell lines that were used in the publications included in this thesis.

Environmentally induced mouse models

Tumorigenesis in environmentally induced models is driven by the exposure of healthy tissue to mutagens. Radiation and chemicals can damage the DNA leading to random mutations. Environmentally induced models are mainly used to

investigate tumors of tissues that form an interface between the organism and the environment like skin, lung and colon (200-202). The length and severity of tumorigenesis can vary between animals. Due to its random nature, environmentally induced tumors are characterized by high degrees of variance, delayed onset and long latencies. Even though it reflects the variability of naturally occurring tumors (203), one has to consider increased numbers of mice for the experimental setup in order to obtain statistically significant results.

Genetically engineered mouse models

The guided introduction of specific mutations, overexpression of oncogenic driver genes or the knock-out of tumor suppressor genes are commonly used to generate genetically engineered mouse models of cancer. The potential benefit of such models is the controlled temporal and spatial tumorigenesis.

We made use of the MMTV-PyMT mouse model of breast cancer. In this model, the mouse mammary tumor virus (MMTV) promoter drives the expression of the polyoma middle T antigen (PyMT) specifically in the breast (204,205). The PyMT antigen assembles several intracellular cell signaling molecules and acts as a scaffold, inducing signaling pathways similar to a constitutively active receptor tyrosine kinase. The constitutive signaling ultimately leads to uncontrolled proliferation and tumor development (206). MMTV-PyMT positive female mice develop several malignant lesions in all ten mammary glands at the age of eight weeks in the FVB/N mouse strain. During the progression the lesions in each gland merge and grow to a palpable tumor. Around the age of 16-18 weeks, mice are usually sacrificed due to impaired movement caused by the primary tumor mass.

Tumors derived from MMTV-PyMT mice classify as luminal-like in early stages and as basal-like in later stages of tumor progression (205). Since tumorigenesis is solely driven by a strong oncogene, there are very few secondary mutations in tumors derived from MMTV-PyMT mice. This does not reflect the heterogeneity observed in the clinic.

In some cases, it is required that a genetic modification is restricted to certain cell types or can be induced at a certain time point, especially if the modification leads to an embryonically lethal phenotype. Conditional systems are used for tissue-specific modifications using the Cre-*lox*P or the Flp-FRT (flippase -*flippase recognition target)* system. Cre removes or inverts genomic DNA target sequences that are flanked by *lox*P sites depending on their orientation. Cell type specific expression of Cre leads to a cell type specific knock-out of the target sequence (207). To generate inducible models, tetracycline or tamoxifen inducible promoters are used to control the expression of Cre. The target sequence will only

be removed when the animal is treated with the inducing drug. Inducible models can be used to knock-out genes that are embryonically lethal after the critical stage of development has passed.

Grafting models

Tumors can be induced by injection of tumor cells or implantation of tumor pieces into host animals (engraftment). Tumor cell suspensions can be injected at different positions. Injections into tissues, other than the tissue a tumor gave rise to, are called ectopic injections. Due to the accessibility and simplicity tumor cells are often injected subcutaneously. Orthotopic injections into the same tissue a tumor is derived from, has the benefit of a matching tumor microenvironment. To study tissue invasion, tumor cells can be injected systemically into the blood stream.

These so-called grafting models are convenient to investigate the effect of single genes *in vivo*. Cultured cells can easily be modified with tools of molecular biology, for example by knocking out/down or overexpressing genes. The generation of a genetically engineered mouse model is more laborious. Transplantation of tumor pieces can be used to increase the number of individuals in the case of rare genotypes.

Syngeneic tumor grafts

A syngeneic graft is the implantation of donor tissues or cultured cells into a host of the same genetic background. Due to the genetic similarity, there is no host-to-graft immune reaction leading to an eradication of the grafted tumor. Therefore, immuno-competent mice can be used for syngeneic tumor drafts.

Xenografts

Grafting of tissues from different mouse strains or other species (xenografts) into immuno-competent mice will cause a host-to-graft immune response. Therefore xenografts require the use of immuno-compromised mice, which lack different parts of the immune system to a different extent.

Nude mice carry a mutation in the *Foxn1* transcription factor gene, causing the lack of hair and the thymus, and were first described in 1966 (208,209). Without a thymus T cells in nude mice do not mature, leading to an impaired adaptive immunity.

Severe combined immunodeficiency (SCID) mice have a deficiency for *Prkdc*, a gene important for DNA repair after homologous recombination during T and B cell development (210). Due to that defect, SCID mice lack an adaptive immunity through B and T cells.

Crossing of SCID mice to non-obese diabetic (NOD) (211) mice resulted in NOD-SCID mice. In addition to the SCID phenotype, NOD-SCID mice also show impaired natural killer (NK) cell function (212).

The removal of a functional IL-2 γ-chain in NOD-SCID mice resulted in the even more immunodeficient NOD-SCID-gamma (NSG) mice. NSG mice are lacking T, B and NK cells and show severe impairment of innate immunity (213).

The severity of the immunodefiency intrinsically defines the type of investigation each murine model is suitable for. Depending on the severity, immunodeficient model systems are not suitable to investigate the interaction of tumor cells with immune cells.

Patient-derived xenografts (PDXs)

After surgical removal of a patient's primary tumor or metastatic lesions, tumor pieces or tumor-derived cell suspensions can be xenografted to generate a PDX. A PDX can be continuously transplanted in sequential generations to amplify the study material or to investigate genetic stability. In early passages a PDX contains the patient's tumor microenvironment, which will gradually be replaced by the host microenvironment in later generations (214).

Cell lines

MDA-MB-231

The MDA-MB-231 cell line is a human breast cancer cell line generated from a 51-year old Caucasian woman in 1973 with a poorly differentiated invasive ductal breast carcinoma (215). MDA-MB-231 cells are hormone receptor negative, HER2 negative and lack expression of tight-junction genes including claudin 3 and E-cadherin, and express high levels of mesenchymal markers. Due to these characteristics, MDA-MB-231 cells are a model for triple-negative breast cancer of the claudin-low subtype (141).

MCF7

The MCF7 cell line is a widely used human breast cancer cell line generated from a 69-year old Caucasian woman in 1973 with an invasive ductal breast carcinoma (216). The cells are ER and PR positive and HER2 negative. Therefore, MCF7 are regarded as a model for luminal A breast cancer. MCF7 cells retain characteristics of the mammary epithelium, displaying an epithelial-like morphology.

BT-20

The breast cancer cell line BT-20 was isolated from a 74-year old Caucasian female. BT-20 cells express an ER variant containing a deletion of exon 5 with constitutive transcriptional activity (217).

4T1

4T1 cells are a series of murine cells isolated from a mammary tumor of a donor mouse of Balb/c background (218). All subtypes of 4T1 cells give rise to primary tumors but metastasize to different locations *in vivo* (219).

EO771

EO771 cells are murine cancer cells isolated from a mammary tumor of a donor mouse of C57BL/6 background (220). EO771 cells are less metastatic than 4T1 cells but display a much faster growth of the primary tumor (221).

Single-cell sequencing

The genetic code is saved in DNA polymers. Deciphering the genetic information has revolutionized the understanding of biology. In the early days, sequencing was expensive and only relatively short sequences were obtained with laborious methods. Since then, sequencing methods improved regarding costs and throughput. Next generation high throughput sequencing-based experiments have become more and more common as the sequencing costs per base dropped dramatically to the point where the storage of the acquired data is more expensive than the acquisition itself (222). The availability of numerous genomes from population based studies, improved our understanding of hereditary diseases and evolution.

Between cells of the same organism, the genome is relatively stable, with the exception of somatic mutations that can occur during each cell division. Even though all cells share a similar genome, each higher organism is made up of several specialized cell types that fulfill different functions. Cell processes depend on the transcription of genes to mRNA and its translation into functional proteins. RNA-sequencing is used to analyze the transcriptome of samples, revealing functional differences.

The transcriptome of a cell changes between each cell type. Even within a cell type, a cell reacts to stimuli such as signaling molecules, drugs or pathogens with specific transcriptomic changes. Therefore, transcriptomics has significantly improved the understanding of cellular processes and functions.

Standard RNA-sequencing protocols require high amounts of RNA, which is therefore isolated from multicellular samples such as tissues, liquid biopsies or cell culture pellets. In the past years, sensitive methods which only require the RNA isolated from a single cell were developed. The analysis of single-cell transcriptomes revealed a previously unappreciated heterogeneity in cell populations (223,224).

Even though protocols were specifically designed for single-cell RNA-sequencing, the method still struggles with some technical limitations. Primarily, the relatively low amount of RNA in individual cells leads to many drop-out events during the preparation of the sequencing library and the sequencing itself. This results in zero-inflated expression data and high variation (225). Other problems, such as the occasional library preparation of double cells, can be solved during the bioinformatics based quality control of single-cell RNA-sequencing data (226).

Workflow

In order to obtain single-cell transcriptomic data, individual cells need to be selected, RNA needs to be isolated and reversely transcribed to cDNA, followed by cDNA amplification and library preparation. Regardless of the protocol used to generate sequencing libraries, the actual sequencing is commonly done on Illumina HiSeq platforms (227).

The isolation of cells from a tissue requires different procedures, depending on the type of tissue. Isolation of CAFs from a breast tumor sample requires rough mincing of the tumor, enzymatic dissociation of the extracellular matrix and gentle straining through a mesh. To obtain optimal RNA yield, dissociation protocols need to be fast yet gentle.

In order to continue the processing and library preparation, single cells need to be isolated. Micropipetting of single cell suspensions or laser microdissection from tissue samples are precise and robust against doublet selection, but time-consuming and lead to many empty wells. Isolation of single cells with FACS is much faster. During FACS, cells can be selected based on surface marker expression, providing metadata for the single-cell transcriptomics analysis (index sorting). However, FACS is sensitive for the selection of doublets and exposes cells to physical stress which can reduce RNA yield and quality. In contrast, microfluidics devices like the Fluidigm C1, ensure the selection of healthy cells but are biased for cell size. Microwell dispensers can spot single cells in wells, but require the inspection by microscopy to ensure cell viability and to exclude the spotting of multiple cells (226).

For the Drop-Seq method, single cells are captured in liquid droplets containing lysis buffer and beads covered with unique primer sequences (228). In the droplet,

mRNAs of single cells bind to individual oligo(dT) sequences within the primers and are therefore barcoded. The barcoded mRNAs can be combined for cDNA and library preparation by dissociating the individual droplets. The pooling and parallel preparation of several transcriptomes reduces costs and increases throughput. However, mRNA binding within the droplet is not as sensitive as other approaches (227).

Oligo(dT) primers are used to capture polyadenylated mRNAs for cDNA preparation in most available protocols. This way most non-coding RNAs will be lost. SUPeR-Seq and MATQ-Seq use primers with random elements combined with fixed sequences to increase the coverage of RNA species (229,230). These primers are designed to exclude ribosomal RNA.

Many protocols rely on polymerase chain reaction (PCR) for the amplification of cDNA. Some methods use 5' attachment of poly(A) or poly(C) sequences as adapters for subsequent amplification. STRT (231), Smart-Seq (232) and Smart-Seq2 (233) use the switching mechanism at 5' end of RNA template (SMART) (234). Despite its common use, PCR-based amplification is biased towards shorter and less GC-rich amplicons. This can potentially change the amount of detected transcript and therefore bias the subsequent analysis. To overcome this bias, CEL-Seq and CEL-Seq2 use *in vitro* transcription to amplify sequences in a linear way (235,236).

Some protocols like SCRB-Seq (237) and Drop-Seq (228) make use of unique molecular identifiers (UMI) and early barcoding. UMIs are small random sequences in each RNA binding primer. The UMIs will be transcribed and amplified together with the mRNA during the library preparation and the sequencing. UMIs can be used to recapitulate whether a sequencing read derives from an individual RNA molecule or from amplification duplicates (238). The use of UMIs therefore increases the accuracy by correcting for the bias introduced by PCR amplification.

Choice of method

The choice of a single cell analysis method is based on the underlying question. Ziegenhain and colleagues analyzed different single-cell RNA sequencing methods regarding their sensitivity, accuracy, precision, power and costs (227). Sensitivity refers to the probability to convert an RNA molecule into a cDNA that is included in the library. Accuracy describes how well the read quantification reflects the actual amount of RNA transcripts in a single cell. Precision identifies the technical variation of a method. The power is calculated using sensitivity, precision and the amount of cells used to draw a conclusion. The authors determined Smart-Seq2 to be the most sensitive method in comparison with

SCRB-Seq, Smart-Seq/C1 (C1 samples were run on a Fluidigm C1 chip), CEL-Seq2/C1, Drop-Seq (228) and MARS-Seq (239). Smart-Seq2 was also shown to be most accurate, but the difference compared to all other methods was negligible in practical terms. The use of UMIs strongly improves the precision; therefore, SCRB-Seq was estimated to have the highest power. When taking the costs of each method into account, Drop-Seq is the most cost-effective method and Smart-Seq2 is among the most expensive ones. Svensson and colleagues published another comparison of single-cell methods and conclude that all available single-cell sequencing methods are highly accurate (240). The choice of the method should therefore be based on the tradeoff between the required sensitivity towards individual reads and quantity of transcriptomes.

We used Smart-Seq2 in our single cell transcriptomic experiment for several reasons. Firstly, we aimed for high coverage and maximum sequencing sensitivity, as we investigated subpopulations of a specific cell type. We assumed that subtle differences between these cells might not be caught using less sensitive approaches. Furthermore, we did not need the high amount of transcriptomes obtained by methods like Drop-Seq, on which the increasingly popular 10x genomic system is based on, since we eliminated contaminating cells by FACS sorting. Lastly, Smart-Seq2 has the advantage that the preparation of the single cell suspension and sorting can be done wherever a FACS machine is available. The lysed single cells can be frozen and prepared for sequencing at different time points or at another location.

The present investigation

Paper I: Cartilage oligomeric matrix protein contributes to the development and metastasis of breast cancer

Aim: Determine the prognostic potential and effects of COMP expression of malignant breast cancer epithelium.

Summary: COMP expression in malignant epithelial cells of breast cancer, in contrast to stromal expression, is associated with decreased cancer-specific survival and recurrence-free survival in two independent patient cohorts. *In vitro* COMP overexpression in MDA-MB-231 cells led to increased invasion and expression of MMP9, but did not change the growth rate, migration, and adhesion. *In vivo,* COMP over-expression in orthotopically transplanted MDA-MB-231 cells, lead to increased tumor growth and metastatic spread to lymph nodes and lungs. Microarray analysis of RNA expression in the experimental tumors revealed an upregulation of gene pathways involved in endoplasmic reticulum stress (ERS) and oxidative phosphorylation. Further *in vitro* characterization in MDA-MB-231 and BT-20 cell lines revealed that COMP over-expression protects against brefeldin A induced apoptosis and reduces the oxygen consumption of tumor cells, the so-called Warburg effect.

Paper II: PDGF family function and prognostic value in tumor biology

Aim: Characterization of PDGF receptor and ligand function based on transcriptomic data from 16 solid tumor types available in The Cancer Genome Atlas (TCGA).

Summary: The PDGF family is involved in cell signaling in malignant disease. Immunostaining of tissue sections from different tumor types for PDGF family members deposited in the Human Protein Atlas (www.proteinatlas.org), indicate a common paracrine signaling route between tumor cells and the mesenchymal stroma, as PDGF receptors are mainly expressed by stromal cells. We used

publicly available RNA sequencing data in order to elucidate gene profiles correlated to each PDGF ligand and receptor chain. We identified functional patterns through gene set enrichment analysis (GSEA) of the correlated data. Our analysis revealed a conservation of functional gene sets in most solid tumor types, related to PDGF signaling. Interestingly, in glioblastoma and renal carcinoma functional profiles differed from the majority of solid tumor types. We detected commonly correlated genes for each PDGF family member and derived gene signatures with improved prognostic value compared to the single PDGF family member.

Paper III: Microenvironmental control of breast cancer subtype elicited through paracrine platelet-derived growth factor-CC signaling

Aim: Deciphering the role of PDGF-CC signaling between breast cancer-associated fibroblasts and malignant cells, and its influence on breast cancer molecular subtype characteristics.

Summary: Immunostainings for PDGF-CC on a breast cancer tissue microarray revealed an association of PDGF-CC positivity in malignant epithelial cells with reduced patient survival. To further investigate the role of PDGF-CC signaling, we crossed MMTV-PyMT mice with a *Pdgfc*$^{-/-}$ mouse. *Pdgfc*$^{-/-}$ tumors were characterized by slower growth, reduced ECM deposition, increased hypoxia, reduced levels of VEGF and increased abundance of necrotic areas. These observations were confirmed by pharmacologic neutralization of PDGF-CC, using the inhibitory antibody 6B3, in an orthotopic xenograft model using the human breast cancer cell line MDA-MB-231.

Transcriptional analysis of *Pdgfc*$^{-/-}$ tumors compared to wild type tumors revealed an upregulation of forkhead box A1 (*Foxa1*) in the *Pdgfc*$^{-/-}$ tumors. *FOXA1* levels in breast cancer transcriptional data, deposited in TCGA was correlated with a non-basal-like molecular subtype. Furthermore, *Pdgfc*$^{-/-}$ tumors with high FOXA1 protein levels also express ERα, exhibiting features of the luminal molecular subtype. We confirmed the association of FOXA1 with the luminal subtype using a panel of breast cancer cell lines and observed an association of PDGFC expression with the basal-like subtype.

PDGFRα, the receptor for PDGF-CC was mainly found on stromal cells, suggesting a paracrine signaling circuit between tumor cells and CAFs. *In vitro* experiments revealed that CAFs respond to PDGF-CC signaling through secretion of stanniocalcin 1 (STC1), HGF and IGF-binding protein 3 (IGFBP3). These three

factors reduce *Foxa1* and *Esr1* (gene coding for ERα) transcription in malignant cells and decrease sensitivity to endocrine treatment *in vitro*, suggesting a shift to a basal-like phenotype (figure 5). Genetic knock-out and pharmacological neutralization of PDGF-CC *in vivo* enhanced sensitivity of basal-like tumors to endocrine treatment and increased the proportion of $ERα^+$ cells in a PDX model of breast cancer. Conversely, PDGF-CC over-expression in grafted luminal-like MCF7 cells reduced sensitivity to endocrine treatment *in vivo*.

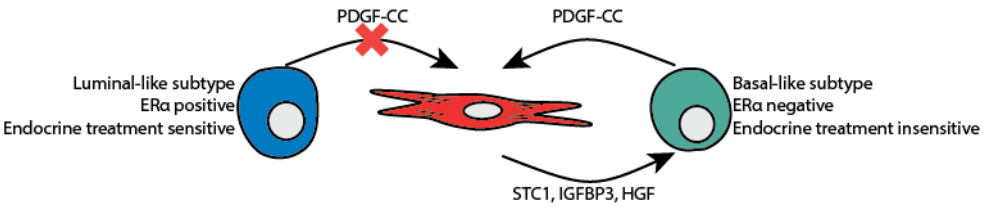

Figure 4:PDGF-CC communication loop
Tumor derived PDGF-CC causes fibroblasts to release STC1, IGFBP3 and HGF which in turn lead to a basal-like subtype, ERα negativity and insensitivity to endocrine treatment. Without PDGF-CC signaling tumor cells display a luminal-like subtype characterized by ERα positivity and sensitivity to endocrine treatment.

Paper IV: Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell RNA sequencing

Aim: Identifying and characterizing CAF subclasses in a mouse model of spontaneous breast cancer, based on single-cell transcriptomic data, followed by assessing the clinical relevance of subclass-derived gene signatures.

Summary: Research on CAFs struggles with the lack of specific marker genes, as several suggested CAF markers are also expressed in varying compositions in stromal cells as well as other cell types. We circumvented this problem by using a negative FACS based selection for $EPCAM^-CD31^-CD45^-NG2^-$ cells. We sorted 768 cells from cell suspensions of tumors derived from MMTV-PyMT mice for single-cell RNA-sequencing, following the Smart-Seq2 protocol. After quality control, 716 single cell transcriptomes remained for further downstream analysis. All transcriptomes contained typical mesenchymal transcripts, confirming the feasibility of the negative selection approach. Dimensionality reduction by principal component analysis and *t*-SNE revealed four subclasses of CAFs with distinct transcriptional profiles. We determined differentially expressed genes in each cluster and derived functional characteristics for each of the CAF subclasses. The first subclass was characterized by genes involved in angiogenesis and vessel development and was therefore termed vCAF. Another class was very similar to

vCAF but was specifically characterized by genes involved in the cell cycle. These cycling cCAFs were not regarded as functionally distinct, but as a part of the vCAF subgroup, which were the only expanding CAF subclass. The differentially expressed genes in the last two CAF subclasses were enriched for ECM and development/differentiation related genes, and were termed mCAF (matrix) and dCAF, respectively.

We validated the existence and spatial distribution of each CAF subclass by immunostaining for suitable marker genes obtained from the differential expression analysis, on tissues of MMTV-PyMT tumors, tumors from grafted cell lines (MDA-MB-231, MCF7 4T1, EO771) and human breast cancer biopsies. Using tissue specimens from different stages of tumorigenesis in the MMTV-PyMT model, we revealed potential different origins of each functional CAF subclass (figure 5). mCAFs were mainly found in the periphery of tumor nodules and displayed typical gene expression patterns of activated fibroblasts, suggesting that mCAF originate from resident breast tissue fibroblasts and were activated by cancer cells. In early tumors, vCAFs were strongly vessel associated and during progression seemed to detach and invade the malignant epithelium. Furthermore, genes detected to be differentially expressed within the vCAF subgroup are also used to define pericytes. Taken together our data suggests PFT as the origin of the vCAFs. Immunostainings of proteins encoded by dCAF specific genes were positive in both epithelial and stromal parts of the tumor. The distribution of dCAF marker expression in the epithelium ranged from uniform to focal hotspots of expression. Of note, the oncogenic driver PyMT of the mouse model used in this study was mainly found to be expressed by dCAFs, suggesting that dCAFs derive from malignant epithelium that underwent EMT.

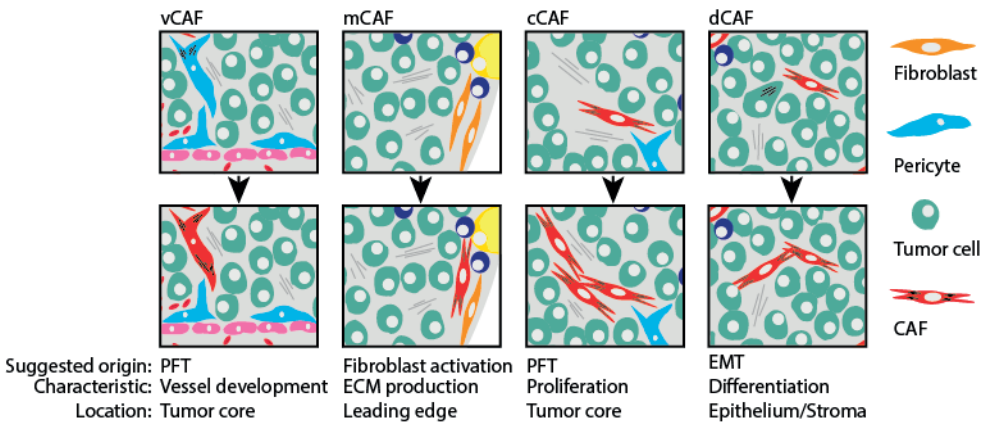

Figure 5: CAF subclasses.
The four detected CAF subclasses have different origins, functions and are mainly found in specific locations.

To determine the clinical relevance of each population, we derived gene signatures for vCAFs and mCAFs, based on correlation of gene expression in human breast cancer data, obtained from TCGA and the METABRIC study. Due to the epithelial expression of some dCAF marker genes, we were not able to identify a signature for these cells. Both vCAF and mCAF signatures were associated to metastatic spread, with a slightly stronger association of the vCAF signature.

Discussion

Paper I

COMP is typically expressed by mesenchymal cells and is mainly located in connective tissues and cartilage. In our study we showed a prognostic value of epithelial COMP expression in breast tumors. COMP expression in TCGA data of breast cancer is correlated to mesenchymal gene expression (cBioportal), suggesting that in most tumors stromal cells are the main source of COMP transcripts. Therefore, expression data from TCGA (bulk RNA-sequencing), accessed on the human protein atlas (241), does not reveal any difference in breast cancer patient survival, which is in line with our observation that stromal COMP expression does not have a prognostic value (242). Our findings underline the importance of spatially resolved data to assess the role of potential prognostic markers in different cell types. In contrast to breast cancer, COMP expression in colon cancer in general was associated to a worse outcome (198). COMP expression is also a prognostic marker for renal cancer, endometrial cancer and urothelial cancer (241).

Stroma-derived COMP did not influence aggressiveness in patients, suggesting that secreted COMP is not responsible for its effect in breast tumors. Indeed, we did not observe signs for changed matrix composition or increased immune cell infiltration upon COMP over-expression, which could be related to the matrix remodeling and complement activation properties of secreted COMP.

The microarray analysis of COMP expressing tumors from our *in vivo* experiments points to intracellular effects of COMP. We observed signs for an altered metabolic profile and reduced sensitivity to ERS. ERS is caused by an accumulation of misfolded protein in the endoplasmic reticulum. Cells react to ERS by activating signaling pathways which are collectively called unfolded protein response (UPR), which is also involved in the development of cancer (243). We showed that COMP over-expressing cells are less sensitive to ERS induced by brefeldin A. TSP4, a member of the thrombospondin family, which is related to COMP, can protect cells from ERS (244). Of note, this function was

dependent on the TIII domain which COMP contains as well. COMP could therefore be able to induce a UPR. Tumor cells commonly produce misfolded protein causing ERS. Therefore, a COMP-induced UPR could lead to a growth advantage in tumor cells.

Liu and colleagues analyzed our microarray data and found an upregulation of the PI3K/AKT/mTOR/p70S6K pathway in COMP over-expressing tumors (198). Furthermore, they observed a reduction of AKT, mTOR and p70S6K phosphorylation upon COMP knock-out in colorectal cancer cells. Of note, mTOR signaling through AKT reduces ERS induced autophagy and apoptosis. Both these processes are common reactions if ERS cannot be resolved (245).

A recent study connected ERS with metabolic changes in cancer-inducing cells, as seen in the Warburg effect (246). They identified the UPR-associated GRP78/p-PERK/NRF2 pathway to mediate the metabolic shift. A link between the UPR and metabolite sensing was proposed before (247). ERS and the changed metabolism are therefore potentially linked in COMP over-expressing cells.

We identified epithelial COMP expression as a prognostic marker in breast cancer. We propose a decreased sensitivity to ERS and an induced Warburg effect to lead to increased aggressiveness of COMP-expressing tumor cells.

Papers II-IV

We investigated the functional similarity of PDGF family members in several solid tumor types (Paper II). We observed that in most solid tumor types the expression pattern of PDGF receptors and ligands between stromal and epithelial cells resembles the expression pattern we observed in breast cancer (Paper III). Mesenchymal stromal cells almost exclusively express PDGF receptors and are therefore recipients of PDGF signaling.

Most detected processes correlating to PDGF expression are commonly present in most tumor types with the exception of glioblastoma multiforme (GBM) and renal cell carcinoma. Subtypes of GBM are driven by alterations in PDGF family members, such as *PDGFRα* amplifications, PDGF-BB over-expression and constitutively active PDGFRβ, underlining the special standing of PDGF signaling in this tumor type (248,249). In renal cell carcinomas expression of PDGFRα and/or PDGFRβ is strongly associated with unfavorable outcome(250,251).

TCGA data indicates that *PDGFC* transcription levels in several cancer types are on average as high as or even higher than in breast cancer. The PDGF-CC induced communication loop between tumor cells and CAFs resulting in the secretion of STC1, HGF and IGFBP3 is potentially active in other tumor types as well. It needs

to be elucidated whether PDGF-CC inhibition in other malignancies could have a beneficial outcome.

The three CAF-secreted factors have common implications in malignant diseases. The HGF receptor c-Met is encoded by the proto-oncogene *MET* and is commonly expressed by cells of epithelial origin. It is involved in embryonic development and tissue repair. When activated through autophosphorylation, c-Met acts as a scaffold for proteins initiating signaling cascades of the MAPK and PI3K pathways, which are involved in the cell cycle regulation (252). In different cancer types, c-Met was found to be amplified or expressed in a constitutively active form, and was therefore identified as a potential target in cancer therapy, leading to the development and approval of c-Met inhibitors (253). c-Met activation in cancer epithelium through CAF-secreted HGF is therefore potentially driving malignization in several tumor types.

IGF signaling pathways induce proliferation, differentiation and inhibit apoptosis. Circulating IGF is sequestered to IGF binding proteins in tertiary complexes (254). IGFBPs are produced in the liver and circulate systemically, in order to regulate IGF signaling. In humans IGFBP3 is the most abundant member of the IGFBP family in the blood stream. IGFBP3 has a much higher binding affinity to IGF than IGF receptors, and therefore indirectly reduces the mitotic effects of IGF binding (255). In addition to its IGF regulating functions, IGFBP3 can translocate into cancer cells and directly exert anti-proliferative and apoptotic actions as a transcriptional regulator (256). Sender and recipient in our proposed PDGF-CC communication loop are in close proximity within the tumor microenvironment. It is more likely that the effects we observed are due to direct IGFBP3 signaling and not the endocrine regulatory function.

The third investigated CAF secreted protein in the PDGF-CC communication loop is STC1. STC1 is a secreted glycoprotein found in high concentrations in tumors (257,258). STC1 interacts with cell signaling pathways like PI3K/AKT and JNK/c-Jun. In colon cancer, STC1 expression increasing invasiveness and metastasis (259).

The molecular mechanisms of STC1, HGF and IGFBP3 reported in general do not overlap. In our model systems however, the factors seem to work mechanistically in the same line. Each single factor decreased ERα expression in *in vitro*, and the effects added up in combinations of the three CAF-secreted proteins. This phenotypic switch of an ER positive to a basal-like phenotype is supported by studies on HGF and IGFBP3. MET expression was shown to be associated with basal-like breast cancer (260). Constitutively active c-MET drives luminal progenitor cells to a basal-like cell fate (261). IGFBP3 is associated with hormone receptor-negative breast cancer and poor survival (262).

The secretome analysis of PDGF-CC stimulated CAFs included further hits besides STC1, IGFBP3 and HGF. To understand the exact mechanism of the phenotype switch, one has to investigate how breast cancer cells integrate all these signals functionally.

Previous studies have shown plasticity between luminal and basal-like carcinomas, and even that both subtypes may originate from luminal progenitor cells (263-265). Of note, BRCA1 mutation seems to drive the development of basal-like cancers from luminal progenitor cells. Together with our results, these findings suggest that the molecular subtypes are indeed not dependent on the cell of origin as their names would suggest, but several internal and external influences like the interaction with the microenvironment or, as indicated in the literature, the mutation of BRCA1.

In Paper III we refer to CAFs as one population engaging in the PDGF-CC communication loop, however in Paper IV we subdivided CAFs into four subclasses using the MMTV-PyMT model system. We showed that mCAFs, vCAFs, cCAFs and dCAFs differ functionally and proposed three different origins for these CAF subclasses. Among the subclasses, mCAFs are most likely the population responding to PDGF-CC signaling, as they express the receptor chain PDGFRα, with the highest affinity to PDGF-CC. *Igfbp3* transcript levels are strongly elevated in mCAFs, whereas *Stc1* expression is lower compared to the other CAF subclasses. *Hgf* expression is generally low in all detected CAF. We isolated the CAFs from breast tumors of intermediate size derived from 14 week old MMTV-PyMT mice. It is possible that the PDGF-CC communication loop was not yet activated in the tumors we used, as the basal-like features in the MMTV-PyMT model are observed in late stages. On the other hand, moderate levels of *Pdgfc* transcription were detected in dCAFs, which we assume to derive from tumor cells undergoing EMT.

The expression of CD146 in stromal cells is linked to sensitivity to endocrine treatment. In a recent study on clinical material, a subset of CD146-negative CAFs was identified. These CAFs were associated with resistance to endocrine therapy in luminal-like breast cancer (266). Indeed, mCAFs, but also dCAFs, are negative for *Cd146* transcripts, while vCAFs and cCAFs are positive. This supports the role of mCAFs in the phenotype switch of breast cancer.

The expression pattern of vCAF markers in tumor tissues suggest, that they most likely derive from pericytes undergoing PFT. PFT is mainly driven by signaling molecules such as PDGF-BB, which binds to PDGFRβ. Even though *Pdgfrb* transcripts can be found on almost all CAF subclasses vCAFs contain the highest levels. vCAFs are therefore main targets of PDGF-BB signaling. Hosaka and colleagues, propose a depletion of vessel-associated pericytes through PFT in tumors (125). In our model system we still observe vCAF marker expression

closely associated to vessels but also in streaks that infiltrate the malignant epithelium. These vCAF streaks originate from blood vessel associated cells and contain proliferating cells (cCAFs). It has to be shown whether these streaks follow a gradient of tumor-derived signaling molecules, potentially caused by hypoxia. It furthermore needs to be elucidated, whether vCAFs are promoting angiogenesis or attenuate vessel growth and induce vessel maturation like pericytes, as many differentially expressed genes in vCAFs are associated with angiogenesis.

Based on tissue staining and gene expression, we hypothesize that dCAFs derive from EMT. In early stages of EMT, epithelial gene expression is still ongoing, and epithelial specific surface markers are still present (13). Therefore, we likely removed those early EMT cells during our strict negative selection. We did not detect subclass specific expression of the EMT factors Snail, Slug, Twist and Zeb1 in our data. The experiment is designed in a way that only mesenchymal cells contributed to the sequencing, therefore an expression of EMT markers in all populations was expected. A slightly increased expression of E-cadherin in dCAFs compared to the other subclasses, however, hints at an epithelial origin.

To compare our CAF subdivision to previously published studies, it is important to note that some studies are based on protein markers and others on gene transcription. Our transcriptomic data is based on mRNA content and does not regard potential downstream regulation of protein translation.

Öhlund and colleagues detected two CAF subgroups by staining tissue sections of PDAC (128), but these two subgroups did not make up the entire stromal compartment in their model system, leaving further CAF subpopulations uncharacterized. Their two subgroups characterized as $IL6^{high}$ αSMA^{low} and FAP^{+} αSMA^{high} do not share similarity with any of our subgroups. Independent of our subdivision, we did not see the association of *Il6*, *Fap* and *Acta2* in our transcriptional data. Only very few cells transcribed *Il6* at all. Öhlund's CAF populations were detected due to their spatial location within the tissue based on protein staining. It is possible that their observations are PDAC specific and their subdivision requires the information of location in relation to the anatomical structures they described.

Li and colleagues subdivided CAFs from human colorectal carcinoma in two subgroups (129). Except *Acta2,* all markers of both their populations are preferentially expressed in mCAF. We tried to subdivide the mCAF subpopulation based on their marker genes, but without success. The data regarding the CAF subdivision presented in this study is questionable, because only 25 single-cell transcriptomes were obtained from several different patients. Some tumor samples only contributed to one CAF transcriptome for the analysis. Based on the low

quality of individual single-cell transcriptomes this study lacks the power to properly define CAF subpopulations.

Su and colleagues described a $CD10^{+}$ $GPR77^{+}$ CAF population associated with tumor formation and chemoresistance by providing a cancer stem cell niche (130). dCAF express intermediate levels of *Cd10* and *Gpr77*. It has been suggested that EMT is associated with features of stemness, and the generation of cancer stem cells (267,268). Indeed, we detected differentially expressed genes *Sox9* and *Sox10* in dCAF. These genes are associated with mesenchymal stem cell differentiation (269,270), this suggests a potential link between dCAFs and cancer stem cells.

Costa and colleagues sorted CAFs from human breast cancer patients based on a FACS panel of CAF markers and detected four subclasses (131). However, before sorting they expanded the CAFs *in vitro*, which can influence their gene expression (271). One of their subclasses was enriched in juxta-tumor tissue and is therefore most likely a resident tissue fibroblast that was not activated yet. Indeed, this population was mainly characterized by low expression of the used marker genes, reflecting a resting state. We excluded healthy tissue during our sample processing and therefore missed such a population. Another population was completely devoid of all markers used in the FACS sorting. Its existence was confirmed in the stromal streaks of tissue samples. There is no transcriptome in our dataset that does not contain transcripts for any of the six markers they used. We therefore did not detect this population either. The two main classes defined by Costa and colleagues are myofibroblastic CAFs of which one has immunosuppressive properties. The main discriminator between these populations is FAP positivity. In our dataset *Fap* transcription does not discriminate between populations, and each CAF subclass contains both Fap^{high} and Fap^{neg} cells. A preliminary supervised separation based on *Fap* transcription did not confirm their findings in our data.

The comparison shows that the CAF subclasses detected in our unbiased approach mainly do not resemble what has been published earlier. This challenges neither previous nor our results and could be due to technical variation or the use of different tumor types and species. We dissected CAF transcriptomes in a depth that has not been done before, and potentially uncovered layers of complexity that were hidden in previous attempts to subdivide CAFs.

In order to evaluate the clinical relevance of the identified CAF subclasses we derived gene signatures based on expression correlation in breast cancer patient data deposited in TCGA. The signature analysis of our CAF subclasses revealed an association to metastatic spread in clinical data. CAFs can degrade the ECM at the primary site, causing tumor cell invasion (114). mCAFs, with their matrix remodeling functions, are more likely able to facilitate such a process. vCAFs potentially act on tumor vessels and facilitate intravasation. Therefore, both

mCAFs and vCAFs potentially act on different steps required for the metastatic dissemination.

It was generally assumed that CAFs in later stages of disease, support tumor growth by several means. Recent studies however, found that depletion of stromal cells in pancreatic adenocarcinoma results in increased aggressiveness and metastasis (272,273). Özdemir and colleagues depleted CAFs by targeting αSMA positive cells. CAF depletion led to an increased invasion of Tregs and therefore reduced immune surveillance. Since all CAF subsets we detected contained a mix of cells expressing αSMA, we are not able to conclude which of our CAF subclasses would lead to such adverse effects when depleted. This study indicates that a general targeting of CAFs might be harmful.

In the study by Rhim and colleagues, the authors depleted both mesenchymal cells and leukocytes, by interfering with sonic hedgehog (Shh) signaling, but drew most of their conclusions on the depletion of mesenchymal stroma. Shh signaling was either blocked by knock-down of Shh or Smo (downstream of Shh) inhibition. Of note, the Smo inhibition was started when first lesions but no tumors had formed. The depletion of stromal cells in early stages promotes tumorigenesis in their model system. Indeed, in an earlier trial with the same compound, they found the opposite trend when treating already formed tumors (274). Based on the assumption that tissue fibroblasts as well as immune cells in early stages mainly exert tumor suppressive functions, this result could be expected. Since patients with pancreatic cancer are rarely diagnosed at such early stages it would be more relevant to investigate the effects of later interference with Shh signaling. Regardless of the shortcomings, this study indicates that CAF targeting might not be beneficial in all stages of the disease, hence requiring a careful evaluation of the time point of treatment.

Conclusion and future perspectives

More and more studies have addressed the complexity of the tumor microenvironment. The understanding of tumor supportive and tumor suppressive mechanisms has grown and was applied to advance targeted therapy. In paper I we describe how a typically mesenchymal gene, expressed in malignant epithelium, increases the aggressiveness of a tumor by affecting the tumor metabolism and the ERS. In paper II, we delineate the general network of processes associated with the PDGF signaling in cancer, whereas in paper III we show how tumor cells and CAFs interact in a PDGF-CC-dependent communication loop, which in our preclinical studies could be targeted to sensitize basal-like breast cancer to endocrine therapy. Finally, in paper IV we subdivide CAFs by identifying and defining subgroups through their individual transcriptional profile into mCAFs, vCAFs, cCAFs and dCAFs. Understanding the actions of these CAF subtypes might lead to the development of new druggable targets. All CAF subclasses are associated to aspects of the malignant disease. mCAFs and vCAFs (and therefore cCAFs) are associated with metastatic spread and dCAFs are associated with the development of cancer stem cells and chemoresistance. Their various origins might help to find ways how to specifically impinge on each subclass.

The progress of sequencing and imaging methods allows us to obtain transcriptional and histological data with increasing resolution and quality. Single-cell RNA-sequencing of tumors provides transcriptional data specifically for each cell type of the tumor microenvironment. The single-cell analysis of tumors, heterogeneously expressing potential marker genes such as COMP, will make the detection of mechanisms of action and the consecutive potential identification of treatment targets much easier. The investigation of cell-to-cell communication within tissues in high-throughput was impossible due to the limited amount of simultaneously detectable proteins in immunostaining and the loss of spatial information in single-cell RNA-sequencing. Methods combining imaging and sequencing are in development and seem promising as transcriptional data will be available coupled with spatial information. Boisset and colleagues made an attempt to investigate the single-cell interactome (the sum of all cell-cell interactions) (275). They partially dissociated tissues to obtain clusters of just a few cells that are in direct contact, and therefore thought to interact directly. After further dissociation of the cell clusters they were able to do single-cell RNA

sequencing of interacting cells, and to draw a map of the interactome. This interactome was based on multiple small cell clusters and might lack a general perspective.

In order to decide whether CAF subclasses are worth targeting, functional experiments like co-injections with tumor cells need to be performed. Several hallmarks of cancer and the different aspects of CAF-tumor cell interaction need to be addressed in these functional studies, and might even change depending on the molecular of breast cancer. A microenvironment based classification could be added to the available classification systems in the future.

The final goal would be the characterization of general CAF subclasses and signatures in all solid tumor types. In general, it seems like a selective targeting of certain CAF subclasses will lead to a more favorable outcome than a targeting of all mesenchymal cells in the tumor microenvironment.

We identified the PDGF-CC based signaling as a target in triple-negative breast cancer. Our promising preclinical experiments will have to prove safe and effective in clinical studies, before the first patients will benefit from this novel treatment option. The main task will be the identification of patients that will benefit from an anti PDGF-CC treatment.

BRCA1 mutation is an interesting marker that might be used to select patients for clinical trials as indicated in the discussion. PDGF-CC inhibition might be a successful treatment for basal-like tumors that originated from a common progenitor and display a certain level of plasticity.

We showed that CAFs are key players in the PDGF-CC communication loop and the phenotypic conversion from basal-like to luminal-like breast cancer. mCAFs are most likely the responsible CAF subclass in this system. The selection process for patients eligible for anti-PDGF-CC treatment could therefore include a screening of the microenvironment. Not only the type of CAF but also the amount and the spatial distribution could be important, as the paracrine signals in this communication loop are limited by diffusion.

In general, it seems like multiple targeting of the tumor and its microenvironment could be a reliable way to treat malignant disease. This would require continuous sampling and analysis of the cancer interactome. Apart from the massive costs and the burden for the patient, such an undertaking would not be possible with today's available techniques

The development of two dimensional single-cell omics and modeling tools from systems biology might reveal ways to fine-tune the network of interactions in a favorable way. It remains to be seen whether personalized treatment will be successful, and if so, whether it will be available for the general public or only a group of wealthy individuals.

Popular science summary

Every cell in the human body interacts with neighboring cells in the tissue, exchanging information by direct contact or by producing compounds that will transmit the information. This very organized communication ensures the function of tissues and the entire body, and also regulates the division of a cell when needed. Besides the external communication, each cell has numerous inner mechanisms controlling that the genome is not damaged during the cell division. A failure of these mechanisms, for example through alterations of the genetic information, can cause a cell to divide in an uncontrolled manner. If this uncontrolled division continues without the detection of the surrounding cells, the so-called microenvironment, or the immune system, these cells can give rise to a malignant tumor.

A tumor requires the other cell types in the microenvironment to grow beyond a certain size. Blood vessels are needed for the supply of oxygen and nutrients, and connective tissue cells provide stability by producing proteins called the extracellular matrix. Malignant tumor cells can take over the intercellular communication system in order to attract those cell types and influence them to support tumor growth. Connective tissue cells in the tumor, called cancer-associated fibroblasts (CAFs), interact with tumor cells in numerous ways.

We discovered that breast cancer cells communicate to CAFs by the signal molecule PDGF-CC, and as a reply CAFs produce HGF, STC1 and IGFBP3. This process mainly happens in basal-like tumors, the most aggressive breast cancer type with limited treatment options. Disruption of this communication with an antibody that is able to block PDGF-CC, increased the response of breast cancer cells and tumors in mouse models to common hormone therapy. We therefore, discovered a potential new treatment option for patients with basal-like tumors.

Even though we addressed CAFs as one single group of cells, we discovered that there are different subclasses of CAFs, by isolating them from mouse tumors and analyzing their functions, through a process called single-cell RNA-sequencing. We grouped 716 CAFs based on the obtained data and identified four different groups of CAFs in breast tumors, that seemingly originate from different progenitors. Each of these subclasses has special functions that can potentially be exploited in the development of future therapies.

We also discovered that tumor cells producing the protein COMP (Cartilage oligomeric matrix protein), usually found in connective-tissue cells, are more aggressive. COMP producing cells lose control of inner mechanisms controlling cell division and their metabolism.

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