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STUDIES ON THE ROLES OF STROMAL CXCL14 IN TUMOR GROWTH, PROGRESSION AND METASTASIS FORMATION

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Karolinska Institutet

Stockholm 2016

Published by Karolinska Institutet.
Printed by Åtta.45 Tryckeri AB.

ISBN 978-91-7676-342-1

Studies on the roles of stromal CXCL14 in tumor growth, progression and metastasis formation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Cancercentrum Karolinska (CCK) föreläsningssal, R8:00, Karolinska Universitetssjukhuset, Stockholm

Fredagen den 17 juni, 2016, kl 09.00

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To my family

“Oändligt är vårt stora äventyr”

ABSTRACT

Cancer consists of several diseases that are characterized by accumulation of genetic and epigenetic alterations that provide cells with certain capabilities to form tumors. Among these acquired capabilities are enhanced invasion that allow cancer cells to escape from the primary tumor, enter the circulation and eventually reach distant tissues where they form metastasis. Breast and prostate cancer are the most common cancers in Sweden with about 9000 new cases diagnosed each year. The major cause of cancer-related mortality is metastatic disease and new treatments interfering with the underlying mechanisms of metastasis are highly warranted.

Enhanced metastasis formation has been shown to occur by reactivation of the developmental program epithelial-to-mesenchymal transition (EMT), regulated by various stimuli, including secreted factors from the tumor stroma. Cancer-associated fibroblasts (CAFs) are the most common stromal cell type that interacts with tumor cells to promote tumor progression and metastasis formation. CAFs have been identified as an important source of EMT-inducing factors including, among others, chemokines. CXCL14 is a CAF-secreted chemokine that promote tumor progression both via autocrine effects on CAFs and paracrine signaling with tumor cells.

The studies in this thesis aimed to achieve a better understanding of the functions of fibroblast-derived CXCL14 in tumor biology and the clinical relevance of this chemokine, with a focus on breast and prostate cancer. The first study explored the molecular mechanisms underlying the protumoral effects of fibroblasts expressing CXCL14. NOS1 was discovered as an intracellular component of CXCL14 signaling in CAFs that maintain their tumor supporting functions. Enhanced oxidative stress in CXCL14-fibroblasts upregulated NOS1 that augmented tumor growth and tumor-infiltration of macrophages. The second study reports that CXCL14 expression in the tumor stroma is an independent negative maker for breast cancer survival. Based on sub-group specific analyses it was shown that the correlation between stromal CXCL14-expression and poor prognosis of breast cancer was more prominent in basal-like and triple negative breast cancers. Interestingly, only stromal expression and not tumor cell expression of CXCL14 correlated with worse survival. In the third study, fibroblast secreted CXCL14 was shown to promote cancer cell EMT, invasion and metastasis, effects directly induced by CXCL14 signaling. Moreover, ACKR2 was identified as a receptor for the orphan chemokine and CXCL14/ACKR2 signaling correlated with an EMT gene expression signature in breast cancer patients.

In general, these studies have uncovered important functions of CXCL14 in both maintaining a tumor-promoting CAF-phenotype, via induction of NOS1, as well as enhancing tumor progression by induction of tumor cell EMT, invasion and metastasis. Furthermore, ACKR2 was identified as a CXCL14-signaling receptor. Clinical relevance of the experimental findings was established by correlations of CXCL14/ACKR2 signaling with EMT and the identification of stromal CXCL14 expression as an independent marker for survival of breast cancer patients.

LIST OF SCIENTIFIC PAPERS

I. **Cancer-associated fibroblasts expressing CXCL14 rely upon NOS1-derived nitric oxide signaling for their tumor-supporting properties**
Augsten M, **Sjöberg E**, Frings O, Vorrink SU, Frijhoff J, Olsson E, Borg Å, Östman A.
Cancer Res. 2014 Jun 1;74(11):2999-3010

II. **Expression of the chemokine CXCL14 in the tumor stroma is an independent marker of survival in breast cancer**
Sjöberg E, Augsten M, Bergh J, Jirström K, Östman A.
Br J Cancer. 2016 May 10;114(10):1117-24

III. **A novel ACKR2-dependent role of CAF-derived CXCL14 in epithelial-to-mesenchymal transition and metastasis of breast cancer**
Sjöberg E, Milde L, Lövrot J, Hägerstrand D, Frings O, Sonnhammer E, Bergh J, Augsten M and Östman A.
Manuscript

Additional relevant articles not included in this thesis

Local and systemic protumorigenic effects of cancer-associated fibroblast-derived GDF15
Bruzzese F, Hägglöf C, Leone A, **Sjöberg E**, Roca MS, Kiflemariam S, Sjöblom T, Hammarsten P, Egevad L, Bergh A, Ostman A, Budillon A, Augsten M.
Cancer Res. 2014 Jul 1;74(13):3408-17

CONTENTS

LIST OF ABBREVIATIONS

ACKR	Atypical chemokine receptor
αSMA	α smooth muscle actin
BMDC	Bone marrow derived cell
CAF	Cancer-associated fibroblast
CSF-1	Colony stimulating factor-1
CTC	Circulating tumor cell
CXCL14	Chemokine (CXC motif) ligand 14
DC	Dendritic cell
DTC	Disseminating tumor cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal transition
FAP	Fibroblast-activating protein
FGF	Fibroblast growth factor
FSP-1	Fibroblast specific protein-1
GDF15	Growth/differentiation factor 15
GPCR	G-protein coupled receptor
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
iDC	Immature dendritic cells
IGF	Insulin-like growth factor
LOX	Lysyl oxidase
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-to-epithelial transition
MMP	Matrix metallo-protease
MSC	Mesenchymal stem cell
NK cell	Natural killer cell
OPN	Osteopontin
PDGF	Platelet derived growth factor
POSTN	Periostin
PTX	Pertussis toxin
ROS	Reactive oxygen species
TAM	Tumor associated macrophage
TEN	Tumor entrained neutrophil
TME	Tumor microenvironment
VEGF	Vascular endothelial growth factor

1 TUMOR PROGRESSION AND METASTASIS FORMATION

1.1 HALLMARKS OF CANCER

Cancer is a multistep process involving genetic alterations, including point mutations, deletions, amplifications and translocations, and epigenetic changes in oncogenes and tumor suppressor genes that affect cellular regulatory systems. These alterations drive the progressive transformation of normal cells into cancer cells, by providing them necessary capabilities for tumor development. Hanahan and Weinberg postulated six acquired capabilities, or hallmarks of cancer, that is shared by most human tumors: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Together, all these capabilities allow cancer cells to survive, proliferate and disseminate[1].

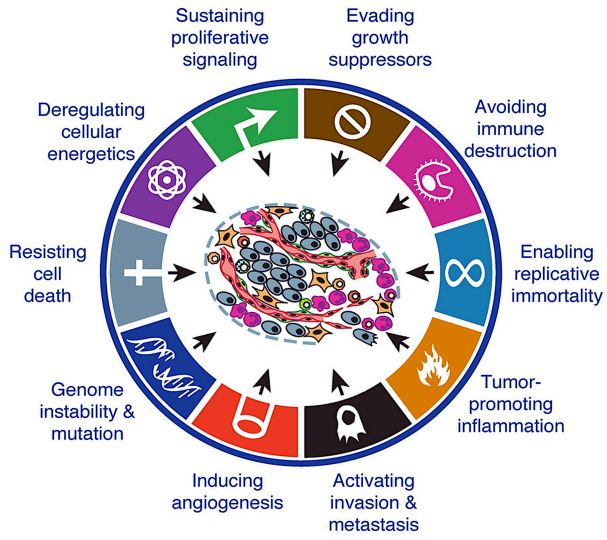

Figure 1: The hallmarks of cancer. Figure modified from[2].

A few years ago, the hallmarks of cancer were revisited and extended (Figure 1). Now, genomic instability, re-programming of energy metabolism, tumor-induced inflammation and escape from immune destruction are recognized as additional hallmarks that contribute and foster tumor development and progression[2]. Interactions with the tumor stroma were also highlighted to contribute to the acquirement of hallmark traits. Cell types and elements of the tumor stroma contribute to several of the hallmarks of cancer, often by paracrine signaling involving secreted factors, as reviewed later.

1.2 THE METASTATIC PROCESS

As the primary tumor grows bigger it invades into the surrounding tissue. Tumor cells disseminating from the primary site enter the circulation and travel with the blood or lymphatic system to distant locations where they form secondary tumors, known as metastases. Like formation of primary tumors, the formation of metastases require the hallmarks of cancer described above but also additional changes, including adaptation to foreign microenvironments and activation of protein degradation[1].

Metastatic disease is the major cause of cancer related mortality. By understanding the underlying mechanisms behind the individual steps of metastasis, formation of new therapeutics can be developed against metastatic disease[3]. A combination of intrinsic programs in tumor cells themselves and the involvement of the microenvironment -both in the primary tumor and the metastatic tissue- are essential for metastatic success. In this thesis, concepts for formation of metastasis, the involvement of the epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) programs in each step of the metastatic cascade will be discussed, as well as the importance of microenvironmental signals for tumor cell EMT, invasion and metastasis to occur.

1.2.1 The "seed and soil theory" for formation of metastasis

Divergent to the hypothesis that spread of cancer cell is only dependent on the vascular anatomy, the "seed and soil" theory for metastatic outgrowth was described by Stephen Paget in the late 19th century[4]. According to this theory, there is a challenge for cancer cells to survive outside the tissue of origin and in order to form metastasis they must find a location with a similar microenvironment. The microenvironment in recipient organs is termed "soil" where tumor cells have a preference to "seed". Thereby, tumor cells have a selectivity to form metastasis in the microenvironment of specific organs, a concept termed "metastatic organ tropism"[5]. For example, breast cancer mainly metastasize to bone, lung, liver and brain, prostate cancer to bone, colorectal cancer to liver, and gastric cancer to lung, liver and the esophagus[6]. Supporting this theory, microarray data have identified genes associated with organ-specific metastatic tropism and metastatic colonization of breast cancer cells to brain, lung, bone or liver[7-10].

This organ selectivity has been shown to involve various factors secreted from stromal cells including chemokines, as discussed below. Most likely, the formation of metastases is likely to result from a combination of the seed and soil theory, and the routes of blood and lymph vessels. The blood flow and lymphatic system directs the journey of the tumor cells but their settlement depends on a suitable microenvironment and appropriate growth conditions at the distant site.

1.2.2 The different steps of metastasis

Metastases are formed as a result of a multi-step process. A reactivation of physiological developmental programs is important for these steps to occur. When primary tumors progress, cancer cells change phenotype, become migratory and promote degradation of the basement membrane extracellular matrix (ECM). They invade into the surrounding tumor stroma and eventually into nearby tissues. This is the first step of the metastatic process, termed "local invasion". During the second step, "intravasation", tumor cells cross the endothelial barriers of blood- and lymph vessels and escape into the circulation. Once in the systemic circulation, circulating tumor cells (CTCs) need to survive before they can disseminate into distant organs. This third step of metastasis formation is denoted "survival in the circulation" or "systemic transport". Only a few malignant cells overcome this hurdle and less than 0.01% of the intravasated cells have been estimated to survive in the circulation[11]. The surviving cells eventually get trapped in vascular beds and migrate through the

endothelium into a distant organ. This process of "extravasation" constitutes the fourth step of metastasis development. During the fifth and last step of metastasis formation, "tissue colonization", tumor cells encounter a foreign microenvironment. As the "seed and soil" theory describes, the formation of secondary tumors requires that tumor cells receive the proper signals to survive and grow in the microenvironment of the distant organ. Hence, only a subset of the cancer cells has the ability to progress and form micro-metastases that ultimately develop into macro-metastases[6,12,13]. Some malignant cells reach secondary organs but in the absence of triggering signals they instead enter a quiescent dormant state[14].

1.2.3 Recent aspects of metastasis formation

In recent years, new aspects of the metastatic process have been revealed which also highlight the importance of the microenvironment. Among these new concepts are the "pre-metastatic niche" and "systemic instigation". The pre-metastatic niche-concept further develops the seed and soil theory and highlights the role of the micromilieu in the establishment of metastases. Systemic signaling, another metastasis-related mechanism, describes one way whereby different cell types of the primary tumor can stimulate the outgrowth of disseminated tumor cells localized at distant sites[15,16].

1.2.3.1 The concept of a "pre-metastatic niche"

The "pre metastatic niche"-model emphasizes the establishment of a niche in distant organs, primed by signals from the primary tumor, preceding the arrival of disseminated tumor cells (DTCs). The establishment of a distant organ-niche is essential to enable DTCs to form a secondary tumor. This is the first model suggesting the involvement of non-malignant cells in determining organ-specific sites for metastasis formation[15,17].

A number of studies have reported the role of bone marrow derived cells (BMDC) in establishing a pre-metastatic niche in lungs. Kaplan et al. showed that subcutaneous lung- or melanoma tumors stimulated fibronectin expression by resident fibroblast-like stromal cells in the lung, which created directional cues for VEGFR-1 positive BMDC and stimulated formation of pre-metastatic clusters. Moreover, BMDC released chemokines, such as CXCL12, that enhanced chemotactic migration of tumor cells expressing CXCR4, which supported the developing metastasis[15]. A more recent study demonstrated that lysyl oxidase (LOX), secreted from breast tumors as a result of hypoxia, is another factor involved in development of a pre-metastatic niche by remodeling of ECM, which promotes recruitment of BMDC into lung tissue[18]. LOX has also been demonstrated to drive the establishment of osteolytic bone lesions that acted as platforms, allowing circulating tumor cells (CTCs) to colonize the bone and form metastasis[19].

A recent published paper reported the involvement of exosomes in the formation of pre-metastatic niches. Hoshimo *et al*. demonstrated that exosomal integrin-expression-patterns determine organotropism. Exosomes from organotropic sublines of the breast cancer cell line MDA-MB-231 were injected in mice prior to injection of tumor cells, with the purpose to educate or prime the organs. Education with exosomes from a lung metastatic subline enhanced the lung metastastatic capacity of a bone metastatic subline. Mass spectrometry identified integrins as mediators of these effects. ITG4 on lung-tropic tumor exosomes

specifically bound fibroblasts and epithelial cells in the lung, and ITG5 on liver-tropic exosomes were taken up by Kupffer cells in the liver. Integrins were also shown to activate Src and S100 signaling that educated the target organ for outgrowth of metastasis. Clinical relevance of these findings was demonstrated by a correlation between high plasma levels of ITGA4 in breast cancer, and ITGA5 in pancreas cancer, and the subsequent development of lung- and liver metastasis, respectively[20].

1.2.3.2 The concept of "systemic instigation"

Another recent concept of metastasis formation is focusing on the systemic signaling that occurs between primary tumors and metastases. McAllister et al. demonstrated that a primary tumor ("instigator") can stimulate the outgrowth of distant, indolent tumor cells ("responders") and named this process "systemic instigation". In their study, nude mice with GFP-positive bone marrow served as hosts for xenograft tumors of breast cancer, and GFP-labeled BMDC were only recruited into responding tumors in the presence of an instigating tumor. Osteopontin (OPN) released in the circulation was shown to be necessary for the recruitment and the subsequent outgrowth of indolent tumors[16]. It is believed that this is a general concept and that other tumor-derived and stromal-derived factors also are important for systemic instigation[16]. Supporting this, growth/differentiation factor 15 (GDF15) was recently described as the first CAF-derived factor that promotes systemic instigation, leading to outgrowth of indolent tumor cells[21].

1.3 EPITHELIAL-TO-MESENCHYMAL TRANSITION

1.3.1 EMT/MET: developmental programs

Organ development during embryogenesis is regulated by conversion of plastic cells between epithelial and mesenchymal states through epithelial-to-mesenchymal transition (EMT) or the reversible mesenchymal-to-epithelial transition (MET). The EMT/MET program is fundamental for different processes during embryogenesis including formation of the placenta, during gastrulation where the germ layers are formed, and formation of the nephron epithelium in the developing kidney[13,22,23].

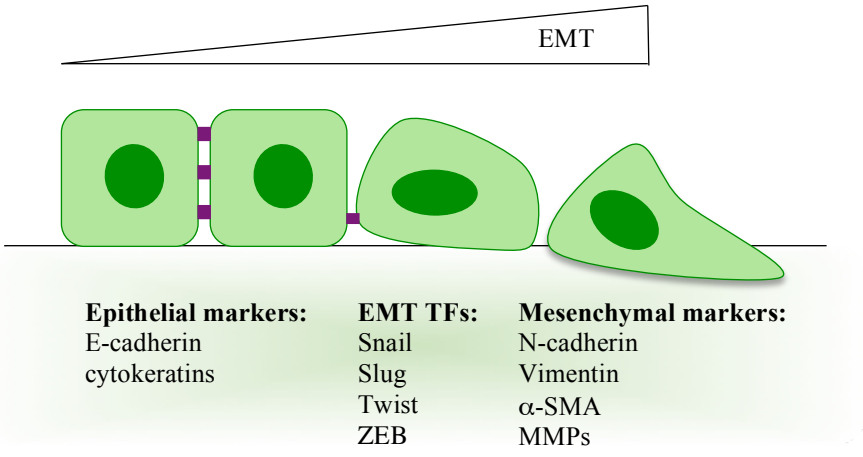

Figure 2: Alteration of molecular markers during EMT.

Functional hallmarks of EMT are loss of cell-to-cell contact, loss of cell polarity, reorganization of the cytoskeleton and induction of a migrating and invading ability of the normally stationary epithelial cells[24]. The cellular and molecular changes are often induced by autocrine or paracrine signaling and are orchestrated by a series of transcription factors, including Snail, Slug, Twist, ZEB1 and ZEB2, that suppress the expression of epithelial markers including E-cadherin and cytokeratins and induce expression of mesenchymal markers including vimentin and α-smooth muscle actin (αSMA) (Figure 2).

The biological processes of EMT and MET are re-engaged in various pathological condition including metastasis formation[13]. In tumors, induction of EMT has been shown to occur in a paracrine manner by secreted factors from cells of the tumor microenvironment (TME), including cancer-associated fibroblasts (CAFs)[24,25]. Among the CAF-secreted factors implied in EMT and metastasis formation are chemokines, further discussed in later sections.

1.3.2 Involvement of EMT/MET in metastasis formation

A re-initiation of the EMT program was previously demonstrated to occur during the early steps of metastasis formation, including local invasion of tumor cells. Nevertheless, the importance of EMT/MET has recently also been demonstrated during later steps, including distant tissue colonization[23]. During MET, tumor cells regain their epithelial phenotypes and enhanced ability to proliferate, essential for the establishment of new tumors at distant sites. The control of the MET program is not well understood but is believed to be regulated by the absence of EMT activation in combination with MET induction. Apart from the enhancement of tumor cell migration, invasion and metastasis, induction of EMT have been linked to stimulation of stem cell properties[22], chemotherapy resistance[26,27] and malignant transformation[28], capabilities not further discussed in this thesis. This section will discuss selected studies, providing clinical and experimental evidence, of EMT/MET during the different steps of metastasis to define the role of EMT in the metastatic cascade.

1.3.2.1 Local invasion

The EMT program promotes several important key changes during the initial step of metastasis formation. EMT is responsible for disruption of cell-to-cell contacts, allow cancer cell to become more motile and enhances the degradation of ECM and basement membrane, processes essential for local invasion to occur. The suppression of E-cadherin has been described as a hallmark of EMT that results from genetic mutations or transcriptional inhibition[29,30].

Several experimental studies have investigated the involvement of E-cadherin loss during invasion and metastasis. Forced expression of E-cadherin in tumor cells and in genetic mouse models was shown to impair tumor cell invasion and metastasis[31]. In a RIP-tag model of pancreatic cancer that maintains E-cadherin expression, spontaneous tumor development was arrested at an early stage. In the same model, expression of a dominant negative mutant of E-cadherin instead induced early invasion and metastasis[32]. The underlying mechanism for enhanced invasion and metastasis as a result of E-cadherin loss was further elucidated in a study by Onder et al. 2008. Knockdown of E-cadherin or expression of a dominant negative mutant enhanced invasion and metastasis of non-metastatic breast cancer cells by

upregulating EMT transcription factors and creating a feed-forward loop for EMT activation[30].

For efficient tissue invasion to occur, EMT also promotes degradation of the basement membrane and adjacent ECM by enhancing cancer cell-production of proteases. The most studied are the matrix metallo-proteases (MMPs) and various reports have revealed upregulation of distinct MMPs by the EMT transcription factors Snail and Slug[33-35]. In addition to paving the way for cancer cells, ECM breakdown also induces release of matrix bound growth factors, cytokines and chemokines that foster cancer cell growth, invasion and metastasis[36,37].

Clinical evidence for the existence of EMT during tissue invasion in human tumors has been confirmed in a number of studies. Loss of E-cadherin has been associated with progression of the disease and poor prognosis in different types of malignancies[38,39]. Gene expression and tissue analyses of human tumors have also given clinical evidence that EMT underlie the metastatic potency of the poor prognosis-associated claudin-low and basal subtype of breast cancer[40,41]. In basal and triple negative breast cancers, tumor cells at the invasive front were also shown to have undergone EMT[42,43]. In a study by Vincent et al, nuclear co-localization of Snail and SMAD3/4 was reported at the invasive front in breast carcinomas. Snail and SMAD3/4 formed a transcriptional repressor complex that repressed epithelial markers during TGFβ-induced EMT[44]. Similar findings have been reported in colorectal cancer where tumor cells at the invasive front, identified by enhanced nuclear β-catenin, displayed upregulated ZEB1, Snail or Twist1[45-47].

1.3.2.2 Intravasation

During intravasation, cancer cells escape through the walls of blood and lymph vessels and are released in the systemic circulation. The EMT program has been proposed to play an essential role in promoting intravasation. In PC3 prostate cancer cells, ZEB1 expression was shown to stimulate transendothelial migration *in vitro* and to enhance metastatic colonization *in vivo* [48].

Analyses of MCF7 cells, using high-resolution imaging techniques, demonstrated the importance of Snail-induced membrane bound MT1-MMP and MT2-MMP for the migration through the vascular basement membrane[34]. In addition, the production of EMT-inducers as TGFβ from endothelial cells and the increased levels of EMT markers in CTCs, as discussed below, further support the hypothesis of EMT-promoted intravasation[49,50].

1.3.2.3 Transport and survival in the circulation

In the circulation, EMT has been proposed to mediate survival of tumor cells and the attachment to vessel walls prior to extravasation. Circulating human and mouse tumor cells have been shown to display expression of EMT markers but the functional effect of EMT in CTCs is still poorly understood[51-56]. Twist1 expression in early lesions of the MMTV-Her2 breast cancer model was associated with elevated number of DTCs in the bone marrow[57]. In concordance, Twist1 induction in an experimental model for squamous cell carcinoma and Snail and Slug expression in a breast xenograft model both enhanced the number of tumor

cells in the circulation. The CTCs displayed an EMT phenotype and the increase was associated with enhanced metastasis[52,58].

One suggested reason for the maintenance of an EMT phenotype in CTCs is the prevention of anoikis, detachment-induced cell death. Loss of E-cadherin in breast tumor cells has been reported to enhance metastasis through induced anoikis[30,59]. As endothelial cells, platelets have been identified as a source for TGFβ-production that might retain the CTCs in an EMT state. A study by Labelle et al. 2011 showed that CTCs associate with platelets and specific inhibition of platelet-derived TGFβ reduced the number of distant metastasis in experimental mice models[60]. In line with these experimental data, CTCs in breast cancer patients have been reported to associate with platelets and to upregulate the TGFβ-pathway[51].

Additional clinical evidence for the involvement of EMT during survival of CTCs comes from observation of EMT-phenotypes in human CTCs. In colorectal cancer, CTCs exhibited EMT phenotypes and the number of CTCs was associated with worse survival[61]. In hepatocellular carcinoma patients, similar findings were demonstrated and the levels of Snail in CTCs were elevated in patients with metastasis[55].

1.3.2.4 Extravasation

The involvement of EMT during extravasation is not well understood and the reason is mainly lack of relevant model systems. The establishment of an extravasation assay in zebrafish, that allow real time imaging of human tumor cells in the circulation, has shed some light on EMT-induced extravasation. In a study by Stoletov et al., forced expression of Twist1 in breast cancer cells was shown to enhance tumor cell extravasation in the zebrafish model. In addition, upregulation of Twist1 also promoted formation of membrane protrusions that enhanced extravasation, essential for and metastasis formation to occur[62].

Interaction with stromal cells has also been reported to affect extravasation. As mentioned earlier, TGFβ signaling from endothelial cells and platelets activated tumor cell EMT that might promote extravasation[50,60].

1.3.2.5 Survival in a new microenvironment and tissue colonization

The relevance of EMT for metastasis formation has been questioned due to the lack of mesenchymal tumor cells present in metastatic lesions. At least two different proposals have been made to explain this phenomenon. Firstly, it has been suggested that mesenchymal and epithelial cancer cells cooperate to complete the full metastatic process. EMT cells may lead the way for invasion and intravasation of non-EMT cells that eventually form the metastastatic tumor[49,63]. Tsuji 2008 demonstrated, by inoculation of mixed labeled mesenchymal and epithelial tumor cells in mice, that mesenchymal cells showed enhanced invasion and intravasation but only the epithelial cells formed the secondary tumors. A second alternative is implying a reversion of the EMT program (or activation of the MET program) during colonization of the new tissue. This is supported by findings that, following tail vein injection of the mesenchymal breast cancer cell line MDA-MB-231, re-expression of E-cadherin was detected in the resulting metastatic lesions[64].

When tumor cells reach secondary organs as the final destination of the metastatic journey, enhanced motility is no longer needed. For tissue colonization to occur, tumor cells instead need to re-initiate proliferation, which has been turned off during the previous steps of metastasis. There is compelling evidence from the literature that proliferation of a cell depend on microenvironmental cues different from those that promote migration, and induction of EMT have been shown to repress cell division and proliferation[52,65-67].

If the absence of EMT-inducing factors in the new microenvironment is sufficient for an EMT reversion or if cancer cells need additional MET inducers is not fully known. Experimental studies have indicated both. Withdrawal of Twist1 activation in DTCs of a skin tumor model enhanced formation of macro-metastasis, indicating that absence of EMT can promote metastasis establishment[52]. In line with these results, loss of the EMT inducer Prrx1 was required for the reversion of EMT in lung tissue, allowing colonization and formation of secondary tumors[68]. The importance of MET-inducing signals has also been shown in the MMTV-PyMT model of spontaneous breast cancer. In this model, bone marrow derived myeloid cells in the metastatic niche of lungs promoted breast cancer cell MET, proliferation and lung colonization by production of versican[69]. The activation of fibroblasts in the lung-niche by metastatic mesenchymal tumor cells has also been reported to induce MET and subsequent tissue colonization, described in detail in the section about CAFs[70].

In summary, this reversible EMT model thus implies that tumor cells can convert between mesenchymal and epithelial states, similar to what occur during embryonic development as discussed above, to be able to adapt to the changing microenvironment both in the primary and metastatic tumor.

2 THE TUMOR MICROENVIRONMENT

Solid tumors are highly heterogenic and composed of several cell types, including cancer cells, endothelial cells, pericytes, various immune cells and cancer-associated fibroblasts (CAFs). There is a tight interplay between stromal and malignant cells, which contribute to cancer initiation, growth, and metastasis, and targeting opportunities within the tumor stroma are continually being identified[25,71]. In addition, the microenvironment in metastatic tissues has recently been reported to play a major role in the establishment of secondary tumors. This section will describe the cells and molecules that form the tumor microenvironment, with relevant examples of studies exploring the function of the primary and metastatic tumor microenvironment in promoting cancer progression, EMT/MET and metastasis. Cancer-associated fibroblasts will instead be separately discussed in chapter 3.

2.1 CELL TYPES AND COMPONENTS IN THE TUMOR MICROENVIRONMENT

2.1.1 Extracellular matrix

Extracellular matrix (ECM) is composed of different molecules that surround the cells in the tumor. These include collagen, fibronectin, hyaluronan, laminin, elastin and proteoglycans, which provide support and anchorage for the adjacent cells and sequester various growth factors. In tumors, degradation of ECM is a requirement for tumor growth and tissue invasion that further promote the release of factors essential for progression and metastasis[72].

Increased matrix stiffness has previously been associated with tumor progression but how the mechanical forces promote the progression of cancer has been elusive[73-76]. In a study by Wei at al, enhanced matrix stiffness was linked to the activation of an EMT response, invasion and metastasis by enhanced nuclear localization of the EMT transcription factor Twist[77]. An increase in stromal collagen deposition has also been shown to correlate with advanced malignancy and metastasis in colorectal cancer and breast cancer, respectively[78,79]. Zhang et al. identified the collagen receptor DDR2 to sustain EMT by stabilization of Snail, which promoted migration, invasion and formation of breast cancer metastasis[79]. Furthermore, hypoxia-induced LOX-expression in primary tumors was shown to enhance ECM remodeling and increase tumor cell invasion and formation of breast cancer metastasis[80]. One alternative mechanism for induction of LOX is transcriptional activation by the ECM component hyaluronan that promoted breast cancer cell EMT, invasion and metastasis by upregulation of twist[81].

LOX has also been shown to be a significant player in the formation of a pre-metastatic niche as mentioned earlier[18,19]. By cross-linking of collagen fibers at distant sites LOX signaling attracted BMDC secreting MMP2. BMDC-MMP2 signaling in lungs enhanced ECM degradation and created a feed-forward loop for BMDC recruitment that established a suitable niche for tumor cells to form metastasis in[18]. The establishment of pre-metastatic niches has also been shown to depend on other matrix components, including Fibronectin[15], Versican[69], periostin (POSTN) and tenascin-C. POSTN and tenascin-C where produced by stromal cells at the metastatic site and activated Wnt and Notch-signaling in cancer cells to facilitate the outgrowth of tumor initiating cells in the lung[82-84]. Together, these studies

highlight the importance of the ECM composition in determining outgrowth of tumor cells at metastatic sites.

A tumor protective effect of the ECM was recently reported in a study on naked mole rats. The naked mole rats are rodents that live underground with an unusual longevity and resistance to tumor development. This study uncovered that secretion of a specific heavy hyaloronan (HA) from fibroblasts was sufficient to make the naked mole rat resist development of cancer. Knock down of HAS2, the enzyme that synthetizes HA, or overexpression of the HA-degrading enzyme HYAL2 made naked mole rat cells susceptible to malignant transformation[85].

2.1.2 Endothelial cells

Tumor angiogenesis is an essential process for tumor growth and metastasis. Progression of tumors is dependent on oxygen and nutrients. As tumors grow, hypoxia and nutrient deprivation tilts the balance between pro- and anti-angiogenic factors that trigger the "angiogenic switch", a transition from an avascularized hyperplasia to a vascularized outgrowing tumor[86,87]. The production of pro-angiogenic factors induce formation of new blood vessels in tumors that exhibit irregular shape and are leaky, compared to normal blood vessels. The most studied angiogenic factor is vascular endothelial growth factor (VEGF). Blood vessel formation is induced when VEGF binds to its receptors on endothelial cells and enhances sprouting and proliferation. VEGF inhibitors are in clinical use for treatment of several cancers[88,89]. In addition to foster tumor growth, the ingrowth of blood vessels also promotes metastatic spread of cancer cells to distant organs[87]. However, anti-angiogenic treatment of tumors have, in some model systems, been shown to promote invasiveness and development of metastasis[90,91]. In this section, the function of endothelial cells specifically during metastatic spread will be reviewed through summaries of selected studies.

Tumor cell-endothelial cell interactions in the primary tumor and at the metastatic site have recently been investigated for the importance of metastasis formation. The involvement of endothelial hypoxia inducible factor (HIF)-signaling for metastatic success was explored in mouse models with an endothelial-specific deletion of HIF1-α or HIF2-α. Loss of HIF1-α signaling in endothelial cells was shown to impede metastasis formation. Injection of GFP labeled Lewis lung carcinoma cells in these models demonstrated that the reduction of metastasis could be linked to impaired intravasation, evident by a reduction of GFP positive cells in the circulation. However, loss of endothelial HIF2-α was instead shown to enhance tumor cell metastasis, demonstrating how endothelial cells can have different impact on metastasis formation[92]. The role of the chemokine signaling during extravasation and metastasis formation of colon cancer was explored by Wolf et al. In this study, CCL2 secreted by tumor cells activated CCR2 and downstream JAK2-Stat5 and p38MAPK signaling in endothelial cells to promote vascular permeability and metastasis formation[93].

The role of the endothelium in regulating breast tumor cell dormancy was also recently explored in *in vivo* models. At breast cancer metastatic sites of brain, lung and bone, dormant tumor cells were shown to reside in the microvasculature. The endothelium constituted a dormant niche that kept cancer cells in a quiescent state through trombospondin-1 signaling. Remodeling of the vasculature and endothelial cell sprouting reduced trombospondin-1 and

induced Periostin and TGF-β1 expression that reverted the tumor suppressing function of the endothelium, which allowed outgrowth of micrometastasis[94]. $VEGFR^+$ endothelial progenitor cells from the bone marrow have also previously been shown to participate in the formation of the pre-metastatic niche, dictating organ specific metastasis[15].

The formation of lymphatic vessels in tumor tissue is believed to occur in parallel with blood vessels, a process called lymphangiogenesis[95], where family members of the VEGF-family play a significant role[96,97]. These vessels consist of specialized endothelial cells -sparsely covered by smooth muscle cells and pericytes- and represents a route for metastatic spread. The number of lymph vessels in tumors has also been shown to correlate with lymph metastasis and poor prognosis in several types of cancers[98-101].

2.1.3 Pericytes

Pericytes are perivascular cells surrounding the blood vessels where they support the vascular wall, regulate blood flow, mediate vessel maturation and remodeling, as well as vascular permeability via paracrine signaling with the endothelium[102,103]. The role of pericytes in cancer is not fully characterized. Some studies have demonstrated that increased pericyte coverage on tumor blood vessels was associated with enhanced tumor growth[104,105]. Others have shown that a reduction of pericyte coverage resulted in an enhanced formation of metastasis[106,107] and correlated with poor clinical outcome[108-111].

It was previously unclear if pericytes actively participated in formation of metastasis or if they only represented a physical barrier to prevent extravasation. One recent study explored the functional role of pericytes in cancer progression and metastasis. By using genetic mouse models and pharmacological inhibitors to deplete or inhibit $NG2^+$ and $PDGF\beta R^+$ pericytes, authors concluded that these cells promote growth of primary tumors but suppress metastasis. The increase in metastasis formation in these mice was explained by an increase in hypoxia, activation of EMT and elevated Met expression in cancer cells. Silencing of Twist or treatment with a Met inhibitor reduced the hypoxic response and the number of metastasis[106].

Different subpopulations of pericytes have been identified based on marker expression and PDGFβR-expressing pericytes was identified as a progenitor cells for different subsets[112-114]. However, the function of individual pericyte-populations in tumor biology is poorly explored. A recent study showed that a subpopulation of PDGFβR expressing perivascular cells in patients with serous ovarian cancer was correlated with worse survival[115], in line with the growth promoting effects of PDGFβR pericytes demonstrated by earlier studies[104,106]. The impact of PDGF-signaling on pericyte-regulation and metastasis development was analyzed in a study by Hosaka et al. In tumors with high levels of PDGF-BB, targeting of the PDGF-pathway inhibited tumor growth and metastasis by preventing detachment of pericytes. In contrast, targeting of tumors with low PDGF-BB ablated vessel-associated pericytes and augmented tumor growth and metastasis[116]. In concordance, several studies have demonstrated that inhibition of PDGF-signaling promoted pericyte detachment and enhanced sensitivity to anti-angiogenic treatment[117-119].

Another study by Keskin et al. further explored the roles of tumor pericytes. The study showed that depletion of pericytes during early breast tumor progression reduced metastasis,

whereas pericyte depletion at later stages of tumor progression was associated with enhanced primary tumor hypoxia and increased metastasis formation. Pericyte-endothelial cell interactions involving angiopoietin-2 signaling were responsible for the increase in breast tumor metastasis, and authors suggested targeting of both pericytes and angiopoietin-2 signaling for treatment of metastatic breast cancer[120].

2.1.4 Platelets

In the circulation, cancer cells must survive various stresses including matrix detachment, the interaction with immune cells and the hemodynamic shear forces. Shielding of CTCs with platelets has been shown to be an efficient strategy to avoid immune recognition, resist the mechanical forces of the circulation and facilitate tumor cell arrest and adhesion to the endothelium[11,121]. By creating a shield, platelets elicit pro-metastastatic functions. However, emerging evidence has also revealed a more complex interplay between platelets and various cell types in the circulation that primes tumor cells for metastasis. Here, a few studies are given as examples of platelet function during the formation of metastasis.

Platelet-tumor cell interaction was shown to enhance tumor metastasis by stimulating EMT, as described previously[60]. Another study by the same authors demonstrated that platelets can guide the formation of an early metastatic niche by paracrine signaling between tumor cells platelets and granulocytes. According to this study, CXCL5/7 chemokine production by tumor cell-activated platelets recruits CXCR2-positive granulocytes to metastatic tissues to form a microenvironment favorable for metastatic seeding[122]. Moreover, Schumacher et al. reported that tumor cell-stimulated ATP secretion from platelets activated $P2Y_2$ receptors on endothelial cells and enhanced transendothelial migration of cancer cells. Abrogation of $P2Y_2$ receptors on endothelial cells or the inhibition of ATP release from platelets in mice prevented tumor cell extravasation and subsequent formation of metastasis[123]. In a mice model of melanoma, the pro-metastatic functions of platelets were shown to be organ specific and platelet-interactions specifically increased lung metastasis[124]. Therapies interfering with platelet function, as long-term treatment with aspirin, have shown success in reducing risk of metastatic disease, which further supports a metastasis-promoting role of platelets[125].

2.1.5 Immune cells

Macrophages, lymphocytes, natural killer (NK) cells, mast cells, granulocytes, neutrophils and eosinophils are immune cells that are present in the inflammatory microenvironment of tumors. Cytotoxic T-cells and NK cells have been shown to target and suppress tumor cells, and infiltration of these immune cells in tumors is associated with a favorable prognosis in several tumor types[126]. During recent years strategies to enhance these cytotoxic responses for the treatment of cancer patients have given promising results. Adoptive cell therapy and the use of monoclonal antibodies against immune checkpoint inhibitors, including CTLA4 and PD1-PDL1, are examples of such strategies[127,128].

On the other hand, mast cells, granulocytes, immature myeloid cells, neutrophils and macrophages are immune cells involved in enhancement of tumor progression, associated with poor prognosis of several cancers[11,129-132].

The following section will discuss a selection of concept-forming literature on two immune cells; macrophages, major producers of chemokines that have been extensively studied for their tumor promoting activities, and neutrophils that recently was reported as major players during tumor metastasis formation by creating a pre-metastatic niche.

2.1.5.1 Macrophages

Macrophages are involved in several oncogenic processes and have the ability to initiate tumor formation, stimulate angiogenesis, promote tumor growth, invasion and metastasis, remodel tissues and regulate immune responses[133,134]. However, the involvement of macrophages in cancer biology is somewhat contradictory. A high infiltration of macrophages has been linked to poor prognosis in several malignancies such as breast, prostate, lung, skin cancer and lymphoma[135-140]. On the contrary, a high intra-tumoral number of macrophages in colon cancer have been associated with a better outcome[141,142]. The explanation for this is a polarization into a "classically activated" tumor inhibitory M1 population, and an "alternatively activated" tumor stimulatory M2 population[133,143].

Various chemokines, such as CCL2, CCL5, CCL7, CCL15, CXCL12 and cytokines including colony stimulating factor-1 (CSF-1), PDGF, VEGF and IL-10, are highly involved in the recruitment of macrophages into primary tumors and metastasis[134,144-147]. Previous studies have suggested that tumor-infiltrated macrophages have a phenotype similar to M2[133,148]. However, recent studies have demonstrated that tumor associated macrophages (TAMs) exhibit a phenotype different from M2, indicating that differentiation of TAMs from monocytes occur through a distinct pathway[149]. In line with these findings, a recent study also revealed that transcriptional activation of macrophages resulted in a spectrum of activation states beyond the M1/M2 phenotypes[150].

The roles of macrophages in tumor cell EMT, invasion and metastasis have been demonstrated by several studies[144]. Some of these studies are here presented as examples. Lin et al showed that silencing of CSF-1 in MMTV-PyMT mice reduced infiltration of macrophages in mammary tumors, which further decreased the formation of pulmonary metastases[151]. Another study revealed the importance of primary tumor-macrophages, via induction of MMP-9 and VEGF, in metastatic colonization in lung of tail-vein-injected tumor cells[152]. DeNardo et al. reported that T-lymphocytes present in breast tumors in MMTV-PyMT mice secreted IL-4 to activate EGF signaling by TAMs. Ligand-activated EGFRs on breast cancer cells stimulated invasiveness, entry to the lung and establishment of metastasis[153].

Macrophage derived factors including chemokines, TGFβ, NFκB, Wnt5a and IL-10 have been shown to induce EMT[154]. A feed-forward loop between macrophages and tumor cells through GM-CSF-CCL18 signaling was demonstrated to activate EMT, invasion and metastasis formation, which is further discussed in the chemokine section[155]. Enhanced CXCL12/CXCR4 and CXCL5/CXCR2 signaling in breast cancer mediated infiltration of GR-1+CD11+ myeloid cells that enhanced invasion and metastasis through upregulation of MMPs and TGFβ[156]. Gao et al. reported that primary tumors of PyMT mice showed an increase in TAMs that created an EMT-promoting microenvironment by production of TGFβ, EGF and PDGF. On the contrary, in the metastatic lesions there were fewer TAMs

and enhanced number of bone marrow derived myeloid cells that instead induced MET by production of the proteoglycan versican[69].

The importance of macrophages in metastatic tumors has just recently been explored. Macrophage-secretion of granulin in liver metastasis of a mouse model of pancreatic ductal carcinoma (PDAC) promoted the shift of hepatic stellate cells into periostin-producing myofibroblasts, which sustained the growth of metastatic tumor cells. Inhibition of macrophage recruitment or granulin secretion reduced stellate cell activation and lowered the metastatic burden[157]. Chemokine-expression in metastatic tissues have also been reported enhance the entry of macrophages. In a mouse model of invasive colorectal cancer, cancer cells secreting CCL9 and CCL15 stimulated chemotaxis of CCR1 positive immature myeloid cells to the liver and enhanced formation of liver metastasis[146]. Another chemokine, CCL2 was shown in a study by Qian et al to correlate with breast cancer metastasis and outcome. The mechanism was explained by CCL2 expression by the target organ stroma and metastatic tumor cells that enhanced recruitment of a subpopulation of inflammatory CCR2-positive monocytes. These monocytes efficiently promoted extravasation and metastatic seeding. By blocking CCL2-CCR2 signaling, metastasis formation was reduced and survival of tumor bearing mice were prolonged[147].

2.1.5.2 Neutrophils

Neutrophils have during recent years been shown to foster tumor metastasis by mechanisms involving establishment of a pre-metastatic niche, enhanced EMT, tumor cell migration and invasion, facilitating extravasation and immunosuppression[158]. Some recent papers discuss the specific involvement of neutrophils at the metastatic site, with different effects on metastasis formation.

Wculek et al showed a recruitment of neutrophils to the lung parenchyma before entry of metastatic tumor cells. Neutrophil production of leukotrienes specifically expanded a subpopulation of tumor initiating breast cancer cells, which eventually formed lung metastasis. Depletion of neutrophils or inhibition of leukotriene production reduced the number of metastasis formed in the MMTV-PyMT breast cancer model and abrogated the pro-metastatic function of neutrophils[159]. Coeffelt et al. reported a cross talk between mammary tumor cells, γδT-cells and neutrophils involving an IL-1β/IL-17/G-CSF signaling cascade. This cascade promoted systemic expansion and polarization of neutrophils that enhanced distant metastasis in experimental models by suppression of CD8 cytotoxic T-cells[160].

As other stromal cells, neutrophils have also been associated with tumor-restraining effects. In the same breast tumor models used as in previously mentioned studies tumor entrained neutrophils (TENs) was shown to have anti-metastatic functions. Consistent with the studies above, TENs arrived to lungs prior to the entry of metastatic tumor cells but was shown to have cytotoxic effects by production of H_2O_2, enhanced by cancer cell-derived CCL2. However, the suppressive functions of these neutrophils were eventually outcompeted by the tumor cells and micro-metastases were formed[161].

3 CANCER-ASSOCIATED FIBROBLASTS

CAFs constitute a major part of many solid tumors and are the most abundant cell type within the tumor stroma, where they are involved in tumor initiation, growth and formation of metastasis. In addition, they display both prognostic significance of different tumors and targeting opportunities[25]. Functions of CAFs, not described here in detail are; support for stem cells[162], immune modulatory effects[163], metabolic interaction with tumor cells[164,165] modulation of drug sensitivity. This section will -with relevant examples of studies- focus on the involvement of CAF-phenotypes in tumor progression and the local and systemic pro-metastatic signaling in both primary tumors and at metastatic sites.

3.1 PHENOTYPES AND ORIGIN OF CAFS

Tumors have been described as wounds that never heal. The tumor stroma is similar to the stroma during fibrosis or wound healing, characterized by an elevated number of fibroblasts, increased capillary density and changes in the ECM[166]. As tumors progress, there is a co-evolvement of the tumor stroma, and fibroblasts exhibit an activated state, similar to fibroblasts associated with wound healing[167]. CAFs display a specific myofibroblast phenotype, are active in ECM turnover and show increased proliferation as compared to normal fibroblasts. Unlike cancer cells, CAFs are not considered to display major genetic aberrations[25,71].

Differences in the expression of cell surface markers suggest the existence of several CAF-subpopulations and it is likely that CAFs in different cancers display functional variations. Markers expressed by CAFs include α smooth-muscle actin (αSMA), fibroblast specific protein (FSP-1), platelet-derived growth factor receptor α and β (PDGFRα and PDGFRβ), fibroblast-activating protein (FAP) and vimentin[71,168].

The occurrence of CAF-subsets can possibly be explained by diverse origins. CAFs have in general been considered to arrive from local fibroblasts stimulated by various growth factors[166]. Experimental studies have provided some additional clues and they might be derived from bone marrow-derived precursors[169-172], arise from normal and malignant epithelial cells that have undergone EMT or from endothelial to mesenchymal transition[173-177]. Pericytes expressing αSMA have been suggested as an additional origin for CAFs[102]. In line with this, a recent study identified a population of activated myofibroblasts during injury, derived from perivascular cells[178].

Emerging multi-marker studies have recently explored the existence of CAF-subsets. A study by Sugimoto et al. could for the first time describe two distinct subsets of CAFs, from models of breast and pancreas cancer. One was defined by expression of αSMA, PDGFRβ and NG2 and the other by expression of FSP1[179].

3.1.1 Transcriptional programs determining CAF-phenotypes

Until recently, very little has been known about the transcription factors that determine CAF-phenotypes. Susan Lindquist laboratory could identify the ubiquitously expressed

transcription factor heat shock factor 1 (HSF1) as an important modulator of reprogramming of resident fibroblast into activated CAFs, which enhanced tumor progression by activating expression of TGFβ and CXCL12. The activation resulted in enhanced angiogenesis, ECM remodeling and increased tumor cell adhesion and migration. The study further demonstrated that stromal expression of HSF1 in lung and breast cancer significantly correlated with worse patient survival[180].

Stromal expression of EMT transcription factors have also lately been shown to alter the CAF-phenotype. Stromal Snail levels was linked to poor prognosis of breast and colon cancer through mechanisms involving augmented ECM stiffness that supported tumor metastasis and altered cytokine production[181]. Another EMT transcription factor, Twist, was described to be involved in activation of CAFs in colorectal cancer and gastric cancer. Twist expressing CAFs induced pro-invasive and pro-tumorigenic effects by increased matrix stiffness and production of secreted factors[182,183]. A study by Sung et al. also showed that Twist1 expression in CAFs was associated with enhanced lymph node metastasis and poor survival of gastric cancer patients[184]. Yet another EMT transcription factor, ZEB1 was demonstrated to distinguish CAFs and normal fibroblasts in prostate cancer[185].

In addition, regulation of other signaling networks have been shown to reprogram CAFs. A transcriptional regulator of hedgehog signaling, FOXF1, was shown to induce a CAF-phenotype significant for progression of non-small cell lung carcinoma[186] and the YAP transcription factor was required for CAF-functions, such as matrix stiffening, invasion and angiogenesis[187]. The vitamin D receptor (VDR) expressed in the tumor stroma of human pancreas cancer was shown to act as a master transcriptional regulator of stromal remodeling, suppressing pancreatic stellate cells (PSC) upon activation with the VDR ligand calcipotriol. The suppression of PSC affected their ability to support tumor growth and a combination of calcipotriol and gemcitabine treatment significantly reduced tumor volume and enhanced survival of treated mice, compared to chemotherapy alone[188].

Epigenetic alteration has also recently been described to enforce conversion of fibroblasts into pro-invasive CAFs, via activation of LIF-signaling. LIF activated an epigenetic switch that enhanced the JAK1/STAT3 pathway and an invasive behavior of tumor cells[189].

3.1.2 Good versus bad fibroblasts in cancer

In vitro experiments of co-cultured normal fibroblasts and cancer cells have previously revealed anti-growth stimulating effects of fibroblasts[190-192]. Lately, also CAFs with tumor restrictive functions have been identified in *in vivo* models of cancer[193-195]

Rhim et al. published that epithelial deletion of Sonic hedgehog (Shh) or pharmacological inhibition of its signaling mediator Smoothened in a mouse model of PDAC reduced the stroma content and enhanced tumor growth, angiogenesis and metastasis[193]. A similar finding that the stromal response to Shh mediates tumor restriction was made in a study on bladder cancer. Stromal deletion of smoothened in mice with chemically induced bladder cancer accelerated tumor initiation, increased proliferation, gave undifferentiated tumors with decreased BMP signaling and reduced mice survival. Activation of BMP signaling prior to formation of invasive carcinoma was able to impede tumor progression by inducing

differentiation of tumor cells[194]. In early and late stage of pancreas cancer, myofibroblasts and fibrosis was also shown to protect against tumor progression, by immune-modulatory effects. Depletion of αSMA-positive fibroblasts reduced survival of tumor bearing mice. Depleted tumors were undifferentiated, showed enhanced EMT and stem cell characteristics. Tumors also displayed an increase in regulatory T-cells but a decrease in infiltration of other immune cells, suggesting that fibroblast restrain tumor progression by enhancing the immune response to control pancreas cancer[195].

3.2 TUMOR PROMOTING EFFECTS OF CAFS

In cancer, CAFs are important for tumor initiation, growth and metastasis[196-199]. Among the pro-tumorigenic factors derived from CAFs are for example growth factors that stimulate proliferation and help to evade apoptosis, factors that induce angiogenesis, factors modulating drug sensitivity and chemokines, mediating various effects on different cell types (Figure 3)[25].

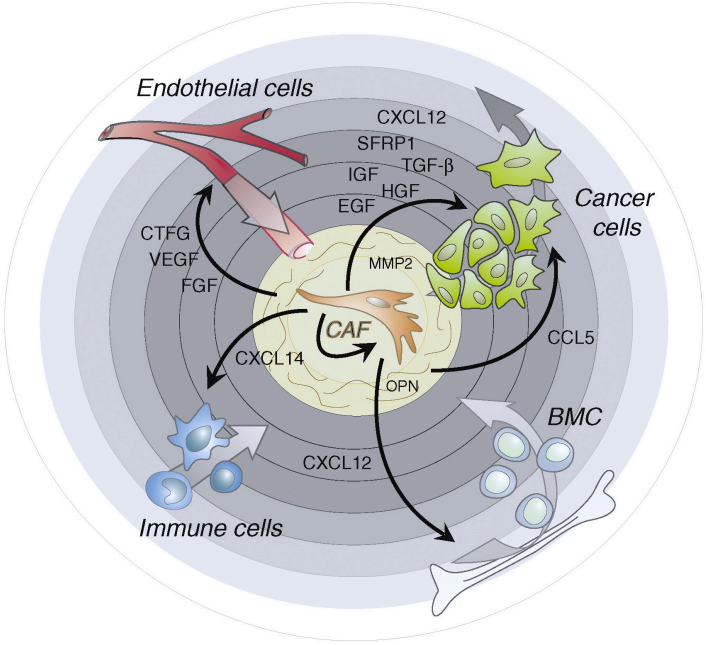

Figure 3: The effects of CAF-signaling on the tumor microenvironment. Figure adapted from[25].

3.2.1 Tumor initiation and growth

CAFs in different tumors produce a variety of factors, including, TGFβ, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), IL-6 and the chemokine CXCL12, that have been shown to induce cellular transformation[196]. A direct tumor initiating capacity of CAFs was shown by a fibroblast specific knock down of the TGFβ type II receptor. The inability of a fibroblast TGFβ response led to spontaneous development of cancer in prostate and the forestomach, and tumor progression through enhanced paracrine HGF-c-Met signaling between fibroblasts and tumor cells[200].

Several other experimental studies relying on co-injection of tumor cells and fibroblasts in immunosuppressed mice have also demonstrated the importance of this cell type for tumor initiation, growth and progression. Some tumor cells only form tumors in mice in the presence of fibroblasts and different fibroblast also vary in their ability to promote tumor growth[201,202]. A study by Erez et al. identified a pro-inflammatory gene signature in CAFs important for their tumor promoting activities, including tumor growth. Co-injection of skin carcinoma cells with CAFs in mice resulted in fast-growing tumors, compared to co-injection with normal fibroblasts. Inhibition of the NFκB signaling pathway revealed its involvement in regulating the pro-inflammatory CAF-phenotype and enhanced tumor growth[203].

Fibroblasts secrete a number of growth factors that enhance tumor cell growth and survival. Epidermal growth factor (EGF), HGF and TGFβ are involved in the sustained tumor cell proliferation, and insulin-like growth factors (IGFs) are involved tumor cell survival[25,166]. CAF-derived factors that stimulate tumor growth also include chemokines, such as CXCL12 and CXCL14 that will be extensively discussed later on.

3.2.2 Tumor angiogenesis

The formation of new blood vessels in tumors -as a result of CAF-signaling- has been shown by direct VEGF-secretion or by induction of other pro-angiogenic factors[71]. These factors include among others, FGFs, CXCL12 and CXCL14[197,204,205]. CXCL12 and CXCL14 are two CAF-derived chemokines that recruit bone marrow-derived endothelial precursor cells or immune cells into growing tumors[197,205]. Fibroblast-derived CXCL14 was highly upregulated in human prostate CAFs and autocrine CXCL14 signaling enhanced angiogenesis through FGF-2 production[205]. Moreover, tumors exhibited increased prostate tumor growth and content of macrophages. The specific role of CAF-produced chemokines will further be discussed in the section about CAFs and chemokines.

3.2.3 EMT, invasion and metastasis

The involvement of CAFs in EMT, invasion and metastasis has been extensively explored. In this section a selection of papers will be discussed that demonstrate conceptual findings regarding local effects of CAFs in the primary tumor, systemically acting CAF-secreted factors and effects of CAFs at metastatic sites.

3.2.3.1 Local effects in the primary tumor

Direct pro-metastatic effects of CAFs in the primary tumor, including effects on EMT, invasion and hypoxia have been shown in several studies.

A pro-metastatic program activated by TGFβ signaling in CAFs was identified in colorectal cancer. TGFβ induced CAF-secretion of IL-11 that bound GP30 on cancer cells and activated STAT3 signaling, promoting the initiation and survival of metastatic cancer cells in the liver[206]. Another CAF-produced factor involved in metastasis of colorectal cancer is the glycoprotein Stanniocalcin 1 (STC1). PDFGRβ signaling was responsible for enhanced expression of STC1 in fibroblasts that increased migration and invasion of cancer cells *in*

vitro. Mice with tumors containing STC1-/- mouse embryonic fibroblasts (MEFs) displayed less metastasis as a consequence of reduced EMT and intravasation[207]. Karnoub et al. also demonstrated that mesenchymal stem cell (MSC) altered metastasis formation through chemokine-induced EMT of breast cancer cells, and the interaction of tumor cells with MSC was required to maintain the metastatic phenotype[199]. The pro-EMT and metastatic effects of chemokines produced by CAFs will be discussed in detail in a later section.

Hypoxia has also been shown to affect CAFs in primary tumors. A fibroblast-specific knock down of HIF-1α in a murine mammary tumor model was shown to accelerate tumor growth[208]. In another study by Madsen et al., deactivation of CAFs was mediated by chronic hypoxia that inhibited PHD2 activity, which prevented HIF1-α degradation and enhanced levels of αSMA and periostin. Pharmacological inhibition or genetic deletion of PHD decreased CAF-activation *in vivo*, diminished matrix stiffness and lowered the number of distant metastasis in a breast tumor model[209]. These data demonstrate how the hypoxic response in the tumor microenvironment can impair tumor aggressiveness by reversion of the CAF phenotype. A study published at the same time reported strikingly similar findings. CAFs isolated from PHD2 haplodeficient PyMT mice showed less activation, impared matrix remodeling and reduced ability to promote invasion and metastasis. A specific haplodeficency of PHD2 in CAFs did however not affect the metastatic ability, and authors instead demonstrated that the above effects was mediated by impaired TGF-β1 signaling from PHD2 deficient tumor cells[210]. Together, these two studies imply that targeting of PHD2 in breast cancer patients could prevent metastatic disease, by affecting both cancer cells and the TME.

3.2.3.2 Systemic effects from the primary tumor and formation of a pre-metastatic-niche

In a recent study, CAFs was demonstrated as a previously unrecognized source of systemic instigation. GDF15 -a member of the TGFβ family- was upregulated in human prostate tumor stroma compared to normal prostate stroma and enhanced levels was shown in the circulation of GDF15-tumor bearing mice[21] and in prostate cancer patients[211]. Forced expression of GDF15 in fibroblasts promoted prostate xenograft tumor growth and, more importantly, enhanced the outgrowth of indolent prostate cancer cells at a distant site in a mouse model for systemic instigation[21]. This is the first study demonstrating a role of the tumor stroma in enhancing metastatic potential by systemic effects.

Pro metastatic factors that have been shown to contribute to the formation of a pre-metastatic niche include members of the VEGF family[15], OPN[16], and LOX[18]. CAFs have been shown to be an important source of these factors that may act systemically to affect the growth of tumor cells at distant sites[71].

3.2.3.3 Local effects in the metastatic niche

The importance of CAF-produced factors in the primary tumor and the metastatic niche, determining metastatic organotropism, was uncovered by the Massagué group. In this study, the abundance of CAFs in triple negative breast cancer patients was linked to the specific establishment of bone metastasis. How these organotropic metastatic traits arose in primary tumors was investigated by analysis of a CAF-gene expression signature from these patients.

CXCL12, IGF-1, CXCL14 and IGF-2 were identified as potential mediators. Experimental data showed that CXCL12 and IGF-1 secreted from CAFs selected tumor cell clones with high Src activity. This selection enhanced the ability of these clones to adapt and survive in the bone marrow -rich in CXCL12 and IGF-1- and eventually to form bone metastasis[212]. Malanchi et al. also demonstrated the importance of CAF-paracrine signaling at the metastatic site. Tumor cells entering the lung interacted with fibroblasts and activated their production of POSTN to initiate tissue colonization[83]. A recent study also demonstrated that POSTN-producing CAFs activated by macrophages in the liver were involved in promoting establishment of liver metastasis, as mentioned earlier[157]. An additional study demonstrated that the EMT/MET program is a key regulator of stromal niche activation at the metastatic site, which enhanced tissue colonization. The mesenchymal phenotype of cancer cells -characterized by AXL expression and EMT markers- and their secretion of thrombospondin-2 were important for activation of fibroblasts in the metastatic tissue. Activated fibroblasts reverted tumor cells from a mesenchymal phenotype to an epithelial phenotype, mediated by inhibition of TGFβ-signaling and induction of BMP-signaling[70].

In summary, CAFs produce a variety of factors that enhance tumor cell invasion and metastasis through an interplay with other cell types in the primary and metastatic tumor, as well as via systemic signaling. In addition, CAFs are also producers of ECM components and matrix remodeling enzymes that increase tumor stiffness and enhance tumor progression and metastasis formation discussed earlier[18,74,187]. Furthermore, CAFs have also been shown to guide cancer cells during invasion, creating paths where tumor cells follow[213].

3.3 CLINICAL RELEVANCE AND TARGETING OF CAFS

3.3.1 Prognostic significance of CAFs

The prognostic significance of single CAF-markers, CAF-derived factors and CAF-gene expression signatures has been demonstrated in a number of studies. A selection of these studies is provided here to exemplify the impact of CAFs on cancer patient outcome.

In breast cancer, stromal marker expression of PDGFRβ, αSMA, TGFβR2 and Gli1 have been identified in various studies to negatively associate with survival[214]. CAF-produced factors correlating with worse breast cancer outcome also include the ECM components hyaluronan and tenascin-C[215,216]. On the contrary, the enhanced levels of FAP in breast tumor stroma was linked to increased disease-free and overall survival[217]. There are also conflicting data from different studies on the impact of certain markers for breast cancer prognosis, which suggest that more extensive research is needed to validate theses as prognostic CAF-markers, and also for the identification of new stromal prognostic markers in breast cancer. These include stromal expression of podoplanin and CAV1[218-222].

Calon et al. discovered that genes previously identified to associate with poor prognosis subgroups of colorectal cancer were enhanced in the tumor stroma compartment compared to the epithelial compartment. Purification of individual cell types from CRC-specimen revealed CAFs as the cell type predominately expressing these poor prognosis genes. Expression of CAF-genes was further analyzed and demonstrated to identify poor-prognosis patients in the good-prognosis subtypes. Stromal expression of the three genes CALD-1, FAP and IGFBP7

were upregulated by TGFβ-signaling and correlated significantly with shorter disease-free survival in colorectal cancer patients. FAP and IGFBP7 also displayed epithelial expression. Notably, the epithelial expression of FAP and IGFBP7 was not associated with colorectal cancer prognosis. Experimental data showed that TGFβ-signaling in CAFs enhanced the tumor initiating capacity of CRC cells inhibition of TGFβ prevented formation of metastasis in mice[223].

Beside the studies on the prognostic role of single CAF-markers and secreted factors, stromal gene expression signatures have recently been explored and shown to correlate with patient survival[223-225]. The evidence for stromal prognostic markers and gene expression signatures also suggest that tumor stroma-characteristics are candidate targets for cancer therapy.

3.3.2 Targeting of CAFs

The tumor stroma has been shown to influence the therapeutic outcome of cancer patients, as well as provide opportunities for targeting. CAFs are likely more genetically stable than cancer cells and thus not as prone to develop resistance to treatment. CAFs can either be targeted by interfering with the pro-tumorigenic secreted factors or by interfering with the recruitment or expansion of CAFs. Considering the importance of growth factors in CAF signaling, as discussed previously, targeting of the recruitment or expansion of CAFs involve inhibition of growth factor signaling. Tyrosine kinase inhibitors, such as Imatinib, Sorafinitinib and Sunitinib that have anti- PDGF receptor targeting activity are used in the clinic for treatment of several malignancies[25,71]. The involvement of chemokines in tumor promoting fuctions of CAFs also suggests these molecules as potential targets.

However, the recent discovery of tumor protective CAF-subsets make targeting of CAFs more complex. Identification of molecular mediators that specifically mediate tumor promoting or tumor restraining effects is therefore prompted.

4 CHEMOKINES

4.1 CHEMOKINES AND CHEMOKINE RECEPTORS

More than 50 chemokines and approximately 20 chemokine receptors make up the chemokine circuit. The large number of chemokines compared to chemokine receptors gives a redundancy within the chemokine signaling network and one chemokine ligand can bind multiple receptors. One receptor can also interact with more than one chemokine [226-228]. Yet, some chemokine receptors only recognize a specific chemokine, for example CXCR4 that only binds CXCL12[226]. Chemokine-ligands are classified based on function, including inflammatory or homeostatic roles, or based on structural motifs. Classical chemokine receptors are named according to which structural ligand-subclass they bind. An alternative class of chemokine receptors does also exist as will be described below.

4.1.1 Classification of chemokines

4.1.1.1 Inflammatory and homeostatic chemokines

Chemokines are divided based on functional properties into inflammatory or homeostatic chemokines. Inflammatory chemokines, as for example CCL2, CCL5 and CXCL8, are upregulated upon inflammation and are involved in recruitment of immune cells to the site of inflammation. On the contrary, homeostatic chemokines including CXCL12 have a constitutive lymphoid organ- or tissue expression and they mediate homing of cells under normal conditions. However, the inflammatory and homeostatic functions are not mutually exclusive and some inflammatory chemokines may have homeostatic roles, and vice versa[227].

4.1.1.2 Structural motifs

The typical chemokine structure involves four highly conserved cysteine residues connected by disulfide bonds. The position of the first two N-terminal-cysteine residues make up the basis for chemokine systematic nomenclature and classification into the four subclasses CXC, CC, CX3C and (X)C[227,228]. The N-terminus of chemokines is important for inducing receptor signaling, but is not critical for high affinity receptor binding[229].

4.1.2 Classification of chemokine receptors

4.1.2.1 Classical chemokine receptors

Chemokine receptors are divided into four classes, named according to the ligand with which they interact. For example, CC chemokines binds CC receptors and CXC chemokines binds CXC receptors. An atypical chemokine receptor class has also been described with distinct functions[227,228,230].

4.1.2.2 Atypical chemokine receptors

The atypical chemokine receptor (ACKR)-subfamily is composed of four receptors designated ACKR1-4. ACKRs are defined as scavenging receptors based on the ability to bind chemokines with high affinity, without activating classical chemokine signaling. They

were therefore excluded from the systematic nomenclature. Two more receptors, CCRL2 and PITPNM3, are under investigation and might in the future be classified as ACKR5 and 6[231-233].

4.2 CHEMOKINE SIGNALING

4.2.1 Classical chemokine signaling

Chemotactic cytokines or, in short, chemokines are a family of proteins secreted by various cell types upon stimulation with inflammatory cytokines, growth factors or pathogenic stimuli. They are involved in several processes, including inflammation, lymphoid organ development, wound healing and cancer, where they influence different aspects of cell behavior, such as cell migration and growth[226,234]. Chemokines are key players in the immune defense against foreign pathogens. One of the best-studied functions is the directed migration of leukocytes toward an increasing chemokine gradient, a process called chemotaxis. Cells expressing the appropriate chemokine receptor will travel toward a high local concentration of the ligand, where they become activated[226,235].

The biological effects of chemokines are mediated by binding to seven-transmembrane-domain G-protein coupled receptors (GPCRs)[229,236]. GPCR-signaling is orchestrated by receptor-associated G-proteins consisting of three subunits; α, β and γ. Upon ligand binding, a conformational change in the receptor enables the substitution of GDP to GTP in the Gα subunit, followed by its dissociation from the G-protein complex. Both the Gα and the Gβγ subunits are released and activates intracellular signaling events[237]. Chemokine receptors belong to the $G\alpha_i$-subfamily of GPCRs and are sensitive to a toxin produced by the bacteria *Bordetella Pertussis*. Pertussis toxin (PTX) catalyzes ADP-ribosylation of the $G\alpha_i$-subunit, which prevents GTP binding and subsequent dissociation and activation of downstream signaling, such as calcium influx, chemotaxis and mitogen-activated protein kinase (MAPK) pathway (Figure 4)[238-240].

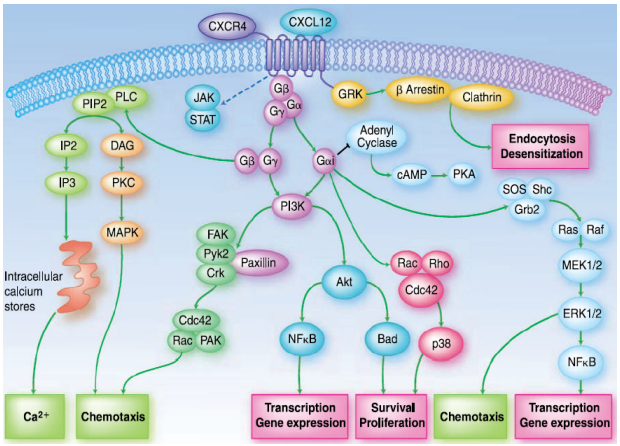

Figure 4: Intracellular pathways activated by chemokine signaling.
Figure adapted from[241].

4.2.2 Signaling of ACKRs

The structural-functional relationships of chemokine receptors are not completely known but certain sequence motifs have been proposed to affect downstream signaling. ACKRs do not all lack the same conserved signature motif that could explain the inability to activate G-protein signaling[242], but modification of the DRYLAIVHA motif has been assumed to explain the inability of atypical chemokine receptors to induce receptor signaling after chemokine interaction[243]. Notably, versions of this signature motif exist within classical chemokine receptors, and the insertion of the DRYLAIVHA motif in ACKR3 did not activate downstream signaling[244].

Recent evidence does also support classical signaling functions for members of the ACKR-family. ACKR3 was demonstrated to bind PTX sensitive $G\alpha_i$-proteins and to activate CXCL12-dependent calcium mobilization, ERK- and AKT signaling in rodent astrocytes and human glioma cell lines. Furthermore ACKR3-CXCL12 signaling in these cell types enhanced their migration and proliferation[245,246].

ACKR2, earlier designated D6 or CCBP2, is an atypical chemokine receptor highly expressed on lymphatic endothelial cells in the skin, gut and lungs, trophoblasts in the placenta[247,248], on macrophages and astrocytes in the brain[249,250]. ACKR2 is mapped to the chemokine receptor cluster on the chromosomal region 3p21, with a DKYLEIVHA version, instead of a DRYLAIVHA motif[251]. The ability to induce calcium mobilization after ligand binding was originally identified for murine ACKR2 by overexpression of the receptor in HEK293 cells[251]. Human ACKR2 did however fail to activate calcium mobilization and chemotaxis in response to the ligand CCL2. Instead, CCL2 interaction mediated receptor internalization and ligand degradation[252].

The inability of ACKRs to induce calcium mobilization or chemotaxis after interaction with certain ligands can be explained by different reasons. ACKRs might have a sequestering function for some chemokines and instead induce signaling for others. ACKRs might also signal via other mechanisms. Recent evidence for a novel β-arrestin-mediated signaling function of ACKR2 was reported in a study by Borroni et al.[253].

4.3 CHEMOKINES IN TUMOR PROGRESSION

In tumors, chemokines and their corresponding receptors have an abundant expression and are produced by tumor cells and stromal cells including leukocytes, endothelial cells and CAFs. They are responsible for the recruitment of various cell types and have been shown to affect tumor growth, angiogenesis, invasion and metastasis[226,254-256]. Nevertheless, the function of chemokines varies among malignancies and they can display either tumor-inhibitory or tumor-promoting roles[226]. In the following sections certain chemokines will be given as examples on how chemokines are involved in tumor biology.

4.3.1 Immune infiltration in tumors

CCL2 and CCL5 are two chemokines that can induce macrophage migration into tumors with subsequent production of macrophage-derived factors that stimulate cancer cell proliferation,

angiogenesis and invasion (discussed in the section about macrophages). In breast cancer and colorectal cancer patients, high levels of CCL5 and CCL2 was correlated with the number of infiltrated macrophages, with an unfavorable prognosis and with lymph node metastasis[257-260]. In tumor models of melanoma, low levels of CCL2 have instead been correlated to an increase of M2 macrophages, blood vessel formation and tumor growth[261]. Correspondingly, the survival rate was higher in pancreatic cancer patients with high levels of circulating CCL2, compared to patients with low levels[262]. These results reflect the contradictory functions of one chemokine in different tumors and the complexity of chemokine signaling.

Other chemokines that contribute to the infiltration of leukocytes in tumors, and with direct pro-angiogenic functions include CXCL8, CXCL1-3 and CXCL5[254,263].

4.3.2 Tumor growth and angiogenesis

Direct effects on proliferation and survival of tumor cells have been shown for chemokines in various cancers. CXCL12 have been shown to stimulate cell proliferation and migration through CXCR4 and ACKR3 (earlier CXCR7)[264-266] and a high CXCR4 expression has been clinically associated with poor prognosis in different tumors[226]. Moreover, CXCL12 has been shown to promote angiogenesis by recruitment of endothelial cell precursors that further facilitates tumor growth and progression[197]. Macrophage-secretion of CXCL8 that interacts with CXCR2 on endothelial cells also induced angiogenesis[267]. Similar findings of CXCL8-promoted angiogenesis related growth and metastasis in mouse models of melanoma and pancreas cancer have also been reported[268,269].

4.3.3 EMT/MET program and metastasis formation

Activation of cancer cell EMT occurs by secreted factors from tumor cells themselves and cells of the tumor stroma[25,172]. Chemokines are example of such EMT-inducing factors as mentioned before. Two aspects of chemokine signaling have been shown to regulate metastasis formation; 1. Local chemokine-crosstalk between stromal cells and tumor cells in the primary tumor that enhance EMT, migration, invasion and subsequently metastasis[155,199,212,270], 2. Systemic effects of chemokines expressed in distant tissues that determine metastatic tropism of tumors cells expressing the corresponding receptors[255,271,272]. The effects of chemokines and chemokine receptors on EMT, invasion and metastasis have been extensively analyzed in experimental cancer models and in human tumors. A few selected studies are discussed below to illustrate modes and mechanisms whereby chemokines influence EMT/MET and metastasis.

4.3.3.1 Pro-metastatic effects of chemokines in the primary tumor

Breast tumor cells that have undergone EMT was shown to secrete GM-CSF to activate TAMs. TAM-production of the chemokine CCL18 enhanced EMT and a GM-CSF-CCL18 feed-forward loop was formed that increased breast tumor metastasis in mice. Moreover, GM-CSF-CCL18 signaling was associated with worse prognosis in breast cancer patients[155]. The chemokine receptor CXCR4 was also demonstrated to enhance breast tumor cell EMT and formation of lymph node metastasis in mice[273]. In a study by Visciano et al. tumor cell-

activated mast cells stimulated EMT and stemness-features in thyroid cancer by activation of a CXCL8-Akt-Slug pathway[274]. In lung cancer cells, CXCR4 and ACKR3 were the most upregulated chemokine receptors by TGFβ. ShRNA-mediated knockdown of the atypical receptor ACKR3, but not CXCR4, reverted TGFβ-induced EMT migration and invasion[275]. A recent study provided further insights of the function of atypical chemokine receptors *in vivo*. ACKR4 (earlier designated CCX-CKR) did not appear to scavenge chemokines in a xenograft model of breast cancer. ACKR4-overexpressing xenograft tumors had no alteration of the ligands compared to control tumors. Instead, the receptor was shown to enhance breast cancer metastasis, via multiple mechanisms that included enhanced motility, EMT and resistance to anoikis[276].

A recent study revealed a molecular link between metastasis and chemo-resistance that involved CXCL1/2 paracrine signaling between tumor cells, myeloid cells and endothelial cells. CXCL1/2 promoted infiltration of myeloid cell into mammary tumors that expressed S100A8/9. S100A8/9 enhanced formation of lung metastasis and further supported survival of cancer cells. Treatment with chemotherapy induced TNFα-production by endothelial cells that enhanced CXCL1/2 expression in cancer cells, which amplified the paracrine CXCL1/2 signaling and caused chemo-resistance. Alteration of this paracrine crosstalk via CXCL1 inhibition increased the efficacy of chemotherapy[270].

4.3.3.2 Pro-metastatic effects of chemokines in the metastatic niche

Chemokines affect tumor progression not just only in the primary tumor site by enhancement of tumor cell growth, angiogenesis, migration and invasion. Also, it has been shown that chemokine expression at metastatic sites will determine the site of metastasis formation. The concept that cancer cells with a certain chemokine receptor will "home" to tissues and organs expressing the chemokine ligand, thereby directing metastatic destination, has been proven for several chemokine receptors. Breast cancer cells was shown express the chemokine receptor CXCR4, which made them home to the bone marrow, lung and liver, tissues where CXCL12 is expressed[255,277]. This concept of chemokine and chemokine receptor involvement in metastasis tropism was confirmed in a B16 mouse melanoma model. By overexpression of chemokine receptors in cancer cells the site of metastases could be controlled. CCR10 was shown to be involved in skin metastasis[278], CCR7 gave lymph node metastasis[279] and CXCR4 caused lung metastasis[280].

Massagué and colleagues could explain how cancer cells survive at distant sites by studying the involvement of chemokines in latent bone metastasis formation from primary breast cancers. In cancer patients, metastasis can occur after several years suggesting a dormant state of tumor cells. In this study CXCL12 was shown to be involved in the survival of dormant breast cancer cells in the bone marrow, which contributed to the metastasis latency[281]. As discussed earlier, CXCL12 derived from BMDC has also been shown to be important for establishment of a pre-metastatic niche in lung[15].

4.3.4 Prognostic relevance of chemokine-signaling

A number have studies have reported on the association of chemokine and/or chemokine receptor expression with patient outcome in different cancers. Worse survival of pancreatic cancer was significantly associated with high CXCL5 expression[282] and hepatocellular carcinoma with high CXCR6 expression[283]. In a study exploring CXCL12 expression in gastric cancer authors identified an upregulation in CAFs compared to normal fibroblasts. Immunohistochemistry (IHC) analysis of human tumor tissue-material showed expression of CXCL12 in αSMA positive stromal cells and an association of high CXCL12 expression and poor outcome. However, no data was shown for the specific correlation of stromal CXCL12 expression and patient survival[284].

4.3.5 Targeting of chemokine-signaling

Small-molecule receptor antagonists inhibit chemokine signaling by interacting with the TM-helices of the receptor. There are two small-molecule chemokine receptor inhibitors on the markets, approved for clinical use. AMD3100 targeting CXCR4 is used for stem cell mobilization, and Maraviroc blocking CCR5 are used for treatment of HIV-1. There are currently no monoclonal antibodies available for use in a clinical setting. However, ongoing clinical trials are promising for treatment of various malignancies. An inhibitor of CCR2 was tested in phase 2 clinical trials for treatment of bone metastases, but was suspended. A CCR4 inhibitor is tested in ongoing phase 1 and 2 clinical trials, either alone or in combination with chemotherapy, for T-cell and NK-cell lymphomas. CXCR4 receptor antagonists are currently in phase 1 clinical trials for treatment of acute myeloid leukemia [285].

4.4 CHEMOKINES AND CANCER ASSOCIATED FIBROBLASTS

As discussed previously in this thesis, the establishment of primary tumors and metastasis is a complex process dependent on the interaction between the malignant cells and the microenvironment. CAFs and chemokines can act independently to promote cancer growth and metastasis, but there is also paracrine chemokine signaling between tumor cells and stromal fibroblasts that enhance tumor progression. In this section, examples of relevant studies will be discussed and a selection of certain chemokines has therefore been made.

4.4.1 Tumor growth and angiogenesis

The research of pro-tumorigenic chemokines produced by CAFs has mainly been focused on CXCL12. The direct effect on tumor cells, such as enhanced proliferation, migration and invasion, mediated via CXCR4 have been shown for several malignancies, including breast-, prostate-, oral- and pancreatic cancer [197,269,284,286-289]. CXCL12 was early shown to be elevated in breast CAFs compared to normal fibroblasts, taken from noncancerous region of the same breast, and was shown to increase growth and blood vessel infiltration of breast tumors *in vivo* by interacting with CXCR4 on malignant cells respectively on endothelial cells[197]. The growth of tumors and angiogenesis was inhibited by treatment with CXCL12 neutralizing antibodies[197]. Furthermore, resident fibroblasts have been shown to engage CXCL12 autocrine signaling to promote the conversion into protumoral CAFs[290].

Primary CAFs was also shown to secrete CCL2 that stimulated breast cancer stem cell characteristics through upregulation of NOTCH1. An inducible CCL2 knock down in primary CAFs was obtained by treatment with doxycycline-induced shRNA against CCL2. Treated CAFs were co-implanted with breast cancer cells orthotopically and administration of doxycycline specifically decreased CCL2 levels, accompanied by decreased NOTCH1 expression and delayed and reduced tumor forming capacity, suggesting that CCL2 is important for fostering the cancer stem cell population in breast cancer[291].

4.4.2 EMT, invasion and metastasis

Chemokines produced by CAFs have also been demonstrated to display pro-EMT, -invasive and -metastatic activities.

In a study by Jung et al. the importance of chemokine signaling for recruitment and activation of CAFs, that further augmented tumor cell EMT and distant metastasis, was reported. In primary prostate tumors, expression of CXCL16 was shown to enhance CXCR6-positive MSC-recruitment and to promote the differentiation into CAFs, based on CAF-marker expression and tumor promoting ability. These CAFs secreted CXCL12 that induced EMT and facilitated dissemination of tumor cells. Knock down of CXCL16 in tumor cells was shown to decrease tumor growth *in vivo* and revert MSC infiltration and CAF conversion[292]. In addition to the involvement of stromal CXCL12 in metastatic organ tropism that was described earlier[212], a role for CXCL12/CXCR4 signaling in enhancing breast cancer metastasis was shown in a study by Smith et al. Inhibition of CXCR4 with RNAi, or the antagonist AMD3100, decreased proliferation and/or survival of malignant cells, and substantially delayed metastatic growth in mice[293].

A study by Qian et al. showed that CCL2 is highly involved in promoting breast cancer metastasis. CCL2 expression both by the lung stroma and by metastatic tumor cells is essential for recruitment of CCR2 positive macrophages, which enhanced extravasation of tumor cell into lung tissue and promoted metastatic seeding[147]. The involvement of CCL5 in metastasis formation has also been demonstrated. GFP-labeled breast cancer cell lines were subcutaneously co-implanted with bone marrow-derived human MSCs in immune-compromised mice. MSCs expressed CCL5 as a response to paracrine stimulation by tumor cells. By interacting with the corresponding receptor CCR5 on breast cancer cells, CCL5 enhanced motility and invasion that further promoted formation of pulmonary metastasis. Inhibition of breast cancer cell CCR5 with shRNA or neutralizing antibodies was sufficient to abrogate MSC-induced metastasis[199].

Together, these studies emphasize the importance of chemokines, produced by CAFs, in affecting both tumor cells and the microenvironment to facilitate tumor growth and metastasis formation. The role of another CAF-derived chemokine, CXCL14 will be discussed in detail in the following section.

5 CXCL14, A PARACRINE PROMOTER OF TUMOR GROWTH

5.1 BIOLOGICAL FUNCTIONS OF CXCL14

CXCL14, also designated BRAK, MIC-1, MIP-2γ or KS1, is a 77 amino acid small protein that belongs to the CXC chemokine subfamily. The chemokine is highly conserved between humans, mouse, birds, frog and fish[294,295]. In humans, CXCL14 is expressed in barrier tissues such as skin, lungs, small intestine, placenta, kidney and brain[296-298]. The chemokine is highly expressed in epithelial cells, but keratinocytes and dermal fibroblasts in the skin, trophoblasts in the placenta and microglia in the brain do all express CXCL14[299]. CXCL14 displays a broad chemotactic activity, as demonstrated for e.g. immature dendritic cells (iDC), monocytes, macrophages, NK-cells and B-cells, but not T-cells[298,300-303]. However, studies on CXCL14 knock out mice have not revealed any alteration in the number of macrophages and dendritic cells (DCs) in the skin, compared to CXCL14 wild type mice. Furthermore, CXCL14 transgenic mice did not display alterations in lymphocytes, macrophages and DCs[299].

Various studies on the biological functions of CXCL14 have identified CXCL14 as a pleiotropic chemokine, involved in immune cell trafficking, glucose metabolism and insulin resistance, neurological functions regulating feeding behavior and neurotransmission, antimicrobial activities and embryonic development[299].

One function of CXCL14 expression in barrier tissues, such as the skin, is antimicrobial effects against certain skin bacteria. Antimicrobial activities are important to avoid extensive growth of microorganisms in constantly exposed tissues. Normally in the skin, this is mediated by specific antimicrobial peptides (AMP) including defencins and cathelicidin LL-37. These peptides mainly mediate receptor-independent effects, but some have also been shown to interact with chemokine receptors. Interestingly, CXCL14 share similar structural motifs as these AMPs and some of CXCL14 functions could thus potentially be receptor-independent[304].

The mechanistic regulation of CXCL14 functions is poorly understood, mainly due to the lack of a known receptor for the chemokine. Identification of a CXCL14-receptor would increase the understanding of the normal biological functions of CXCL14, but also the role(s) of CXCL14 in tumor biology.

5.2 CXCL14 IN CANCER

The expression of CXCL14 is absent in many cancer cell lines and lost in epithelial cells of several tumors[296,297,305,306]. Nevertheless, some tumors show increased expression of CXCL14 in the tumor compartment[307,308] or in the tumor microenvironment[205,277,297,301], compared to normal tissue. The functional role of CXCL14 in various cancers has been addressed in previous reports. Some of them indicate a tumor suppressive function, whereas other instead point to a tumor promoting role of CXCL14[299].

5.2.1 Tumor-suppressive functions of CXCL14

The downregulation of CXCL14 in malignant cells in certain cancers have been shown as a result of epigenetic silencing of the *CXCL14* gene[305,309,310]. In lung cancer patients, the CXCL14 gene promoter is commonly methylated, consistent with enhanced promoter methylation of CXCL14 in lung cancer cell lines. To investigate the effect of CXCL14 silencing during tumor progression, CXCL14 was re-expressed in lung cancer cells. Forced expression reduced tumor growth *in vivo* and enhanced tumor necrosis. *In vitro* data showed a decrease in cell proliferation, increased cell death and interestingly, enhanced cell migration of lung cancer cells[305].

Moreover, studies analyzing the effects of CXCL14 over-expression in cancer cells have reported both anti-tumoral effects through inhibition of cell proliferation in breast cancer [311], and by regulation of immune cells[302,312]. Overexpression of CXCL14 in a highly metastatic subclone of MDA-MB-231 cells reduced proliferation and invasive properties *in vitro*, and attenuated growth of orthotopic xenograft tumors and pulmonary metastasis formation *in vivo*[311].

Since CXCL14 is a chemoattractant for various immune cells, the epigenetic silencing of the gene in malignant cells could be one way of tumors to escape immune recognition. This notion was supported from studies on CXCL14 transgenic mice where chemically induced colorectal cancer was suppressed via a reduction in NK-cell mediated immune surveillance[313].

Chemokines can be classified by the ability to induce angiogenesis based on the presence or absence of an ELR motif [314]. Most of the CXC chemokines are ELR^+ angiogenic factors, with the exception of CXCL12. CXCL12 induces formation of new blood vessels although the chemokine lacks the ELR motif, by effects on VEGF signaling[197,315]. Anti-angiogenic effects of cancer cell- or host-derived CXCL14 have been revealed in animal models of lung cancer, melanoma and head and neck cancer[301,316]. Shellenberger et al. demonstrated that CXCL14 inhibited endothelial cell migration and thereby caused a decrease in angiogenesis, using a rat corneal micropocket assay. This study also detected CXCL14 in stromal fibroblasts next to tumor cells in patients with human squamous cell carcinoma of the tongue. Functional significance of the stromal expression was not analyzed in this study[301].

5.2.2 Protumoral effects of CXCL14

Protumoral effects of CXCL14 have also been identified. In prostate cancer Schwarze et al. showed enhanced *CXCL14* mRNA expression in stromal fibroblasts compared to tumor cells, and stromal levels increased with prostate cancer stage[307]. Also in human prostate and breast cancer, CXCL14 expression was up-regulated in CAFs as compared to normal fibroblsts. As detailed below, the protumoral effects of CXCL14 include increased cell proliferation, invasiveness and stimulation of tumor growth and metastasis.

Tissue culture and mouse cancer model-studies of breast and prostate cancer demonstrated protumoral effects of CXCL14 expressed by stromal fibroblasts[205,277]. Laser capture micro-dissection of matched prostate tumor and non-tumor stroma revealed an upregulation of CXCL14 mRNA and protein in CAFs. Engineered fibroblasts overexpressing CXCL14

promoted an activated fibroblast-phenotype with enhanced proliferation and migration. CXCL14-fibroblasts also enhanced proliferation and migration of LNCaP prostate cancer cell *in vitro* through paracrine effects involving CXCL14-induced factors. Tumor progression was stimulated by CXCL14-fibroblasts through multiple mechanisms, including autocrine effects on the fibroblasts and paracrine stimulation of macrophage infiltration and angiogenesis, without any change in the epithelial-stroma ratio (Figure 5). An increase in NK-cells, as demonstrated in other tumor models, was not detected in this study[205].

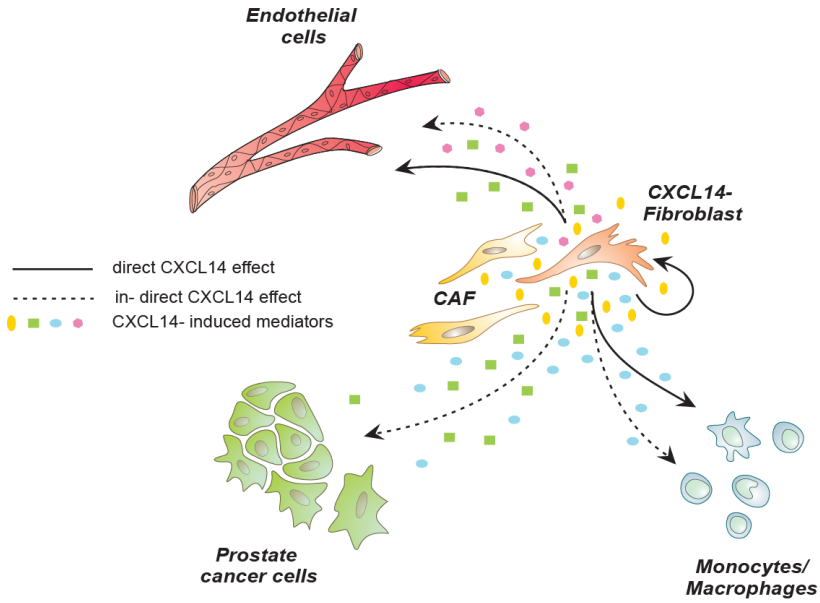

Figure 5: Tumor promoting effects of CXCL14-expressing fibroblasts. Figure adapted from[205].

As CXCL12, CXCL14 is also defined as an ELR$^-$ chemokine and has, as previously mentioned, been demonstrated to exert inhibitory effects on blood vessel formation. However, this study showed that tumors with CXCL14-overexpressing fibroblasts had an increase in CD31-positive blood vessel compared to control tumors, associated with increased levels of FGF-2 released from the fibroblasts. These blood vessels also exhibited a reduction of pericyte coverage[205]. Another study on breast cancer demonstrated migratory and invasive features of stroma-derived CXCL14[277]. Together, these studies suggest protumoral effects of CXCL14 expressed in the tumor stroma across cancer types.

Tumor promoting functions of cancer cell-derived CXCL14 has also been reported. CXCL14 expression was mainly localized to the invasive front of pancreas and colorectal tumors, and the chemokine was also shown to enhance the invasive capacity of cancer cells *in vitro* [308,317,318]. Preliminary findings of E-cadherin downregulation and MMP9 induction in pancreatic cancer cell lines, subsequent to CXCL14 stimulation, have been suggested as an underlying mechanism for the enhanced invasion[308]. Similar findings on altered motility and invasion was found in breast cancer cells through a reactive oxygen species (ROS)-mediated upregulation of CXCL14. Enhanced ROS levels in breast cancer cells augmented CXCL14 expression and promoted migration, invasion, tumor and metastasis formation *in vivo*. ROS activation of the transcription factor AP1 was shown to bind the CXCL14 promoter and enhance Ca^{2+} levels in the cytoplasm by interacting with the inositol 1,4,5 triphosphate. These data reveal a novel activation of an AP1-CXCL14-calcium pathway activated by oxidative

stress in breast cancer that promotes tumor cell migration, invasion and metastasis[317]. In line with this study, another report showed effects of CXCL14 on metastasis formation of osteosarcoma cells *in vivo*[319].

Organ-specific tropism of metastasis has also been linked to CXCL14. A study by Takiguchi et al. showed that CXCL14 was specifically involved in bone tropism of lung cancer cells. A bone-seeking subclone of lung cancer cells upregulated 72 genes, among which CXCL14 was one of the most induced. Knock down of CXCL14 in the bone-metastatic subclone prior to intracardial injection slightly reduced metastasis to the bone and interestingly, increased metastasis to the adrenal gland. Of note, IHC stainings of CXCL14 in CXCL14-silenced tumors showed stromal expression of the ligand, that could possibly also enhance formation of bone metastasis and might explain the non-complete blockade of bone metastasis following CXCL14-downregulation. Furthermore, CXCL14 was highly expressed in bone metastasis from human lung cancer patients, both in the tumor cells and in the bone microenvironment[320].

Another study also demonstrated CXCL14 expression to be elevated in CAFs in ER negative and triple negative breast cancer patients. In addition, CXCL14 was highly expressed in bone metastasis compared to metastasis in lung liver and brain. These correlative data thus suggest that CXCL14 expressed by CAFs in primary tumors also could be important for bone metastatic tropism of tumor cells in certain breast cancer patients[212].

5.2.3 CXCL14 expression and cancer patient prognosis

Only few studies have so far investigated the potential prognostic value of CXCL14 expression in cancer. Some of these analyses demonstrated a correlation between high CXCL14 protein levels and a more favorable prognosis, whereas others showed that CXCL14 was associated with impaired survival[311,321,322].

The *CXCL14* gene was included in two gene expression signatures that predicted worse disease free survival of prostate and ovarian cancer, respectively [321,322]. In addition, *CXCL14* was part of a gene expression signature that correlated with the presence of metastasis in breast cancer patients[323]. Elevated CXCL14 transcripts have also been demonstrated in papillary thyroid carcinoma (PTC) to significantly associate with lymph node metastasis[324].

Analyses of CXCL14 protein levels have also revealed a correlation to cancer survival in several reports. CXCL14 was shown to predict decreased overall survival in osteosarcoma patients and autocrine CXCL14/NFκB-signaling was induced by hypometylation of the gene encoding the transcription factor Iroquois homeobox1 (IRX1). In addition, higher levels of CXCL14 were found in patients with lung metastasis, compared to patients without[319]. Consistently, Zeng et al. showed that an upregulation of CXCL14 in colorectal cancer patients was associated with tumor-node metastasis (TNM) stage, differentiation grade and tumor size, and correlated with disease recurrence and shorter overall survival[318].

Other studies have instead reported on associations between high CXCL14 protein expression and good prognosis in breast and colorectal cancer[311,325]. Notably, none of these studies have considered the cell type responsible for CXCL14 expression and addressed the possibility

that the prognostic impact of CXCL14 may be determined by the tumor compartment in which CXCL14 is produced.

5.2.4 CXCL14 as an inducer of a protumoral CAF-phenotype

Tumor-stimulatory abilities and higher proliferative rates characterize CAFs, as compared to normal fibroblasts[326,327]. As previously discussed, CXCL14 promoted NIH-3T3 fibroblast proliferation and migration. CXCL14-fibroblasts where also more potent in enhancing xenograft tumor growth compared to control fibroblasts, suggesting that CXCL14 could be involved in determining a specific tumor promoting CAF-phenotype[205].

As mentioned previously, expression of the transcription factor twist in CAFs enhanced protumoral effects of fibroblasts. Interestingly, in the study by Sung et al. a Twist-induced CAF-phenotype exhibited increased expression of CXCL14 and FSP-1[184], further supporting expression of CXCL14 in a previously defined CAF-subset[179]. In addition, during liver injury hepatic stellate cells become ECM-producing myofibroblasts, with some phenotypes similar to CAFs. Interestingly, CXCL14 was identified as a novel gene specifically upregulated in these hepatic stellate cells upon activation[328].

Together, these studies indicate that CXCL14 could be a potential marker of a particularly aggressive CAF-subset, a suggestion that need further experimental validation.

5.2.5 CXCL14, an orphan chemokine

Since the receptor for CXCL14 is currently unknown, the downstream signaling of the chemokine is poorly characterized. Calcium influx and activation of NFκB and ERK have been demonstrated as intracellular signaling events of CXCL14[205,329].

The conflicting data on the role of CXCL14 in tumor biology might be explained by the different settings and models used in the different studies, which might display variable expression of the unidentified receptor(s) and tissue- or cell-type-specific signaling. Tumors in which CXCL14 stimulates growth might be characterized by a stromal-specific expression of a CXCL14 receptor. The interaction with CXCL14 could activate fibroblasts and induce secretion of tumor promoting factors that enhances tumor progression. On the other hand, tumors in which CXCL14 is growth-inhibitory might display preferential expression of the receptor on the malignant cells.

Although no signaling receptor has been discovered, CXCL14 has been demonstrated to bind CXCR4 and function as an inhibitor of CXCR4-CXCL12 signaling[330]. However, a recent study showed that CXCL14 did not affect CXCR4 mediated calcium mobilization, MAPK signaling or CXCR4 internalization[331]. This suggests that the functional interaction of the CXCL12 and CXCL14-pathways depend on a yet unidentified receptor and could possibly involve heterodimerization of CXCR4 and this receptor.

An improved understanding of normal functions and roles in tumor biology of CXCL14, as well as rational targeting, is obviously dependent on identification of critical signaling cell surface receptors.

6 PRESENT INVESTIGATION

6.1 AIMS

The general aim of this thesis was to understand the functional roles of the chemokine CXCL14, produced by cancer-associated fibroblasts, in tumor progression and metastasis formation of prostate- and breast cancer, and to investigate the potential clinical relevance of CXCL14. Specific aims were:

- To investigate the molecular cell signaling mechanisms underlying the protumoral functions of CXCL14-expressing CAFs
- To explore the prognostic significance of CXCL14 expression in breast cancer
- To explore potential pro-metastatic effects of CXCL14 produced by CAFs
- Identify a receptor for CXCL14

6.2 RESULTS AND DISCUSSION

6.2.1 Paper I

Cancer-associated fibroblasts expressing CXCL14 rely upon NOS1-derived nitric oxide signaling for their tumor-supporting properties

Cancer-associated fibroblasts are the most abundant cell type within the tumor microenvironment and they stimulate tumor growth, progression and metastasis through paracrine interactions with cancer cells or other stromal cells. The existence of CAF-phenotypes with different abilities to promote or inhibit tumor progression has recently been highlighted. CAF-phenotypes could potentially be identified by distinct markers and secreted proteins. Various secreted factors, including chemokines, have also been identified to promote tumor progression and metastasis. The chemokine CXCL14 was previously identified as a CAF-secreted factor that enhanced prostate tumor growth via multiple mechanisms, including autocrine effects on fibroblasts, and paracrine effects on angiogenesis and macrophage infiltration. In this study, the mechanisms of the protumoral effects CXCL14-expressing fibroblasts were explored and how the tumor promoting features of these CAFs are maintained.

We identified nitric oxide synthase 1 (NOS1) as a novel component of CXCL14-intracellular signaling in CAFs important for their tumor promoting functions. Gene expression analysis of engineered fibroblasts with a stable CXCL14 expression (CXCL14-fibroblasts) revealed an upregulation of the enzyme NOS1, compared to control fibroblasts. The upregulation was induced by enhanced oxidative stress in CXCL14-fibroblasts and the increased NO levels where used in intracellular processes to enhance CAF-tumorigenic functions. This was evident by enhanced protein nitration but no increase in NO-secretion by CXCL14-fibroblasts.

Both genetic and pharmacological inhibition of NOS1 demonstrated a reduction of CXCL14-fibroblast proliferation and migration. Specific downregulation of NOS1 in CXCL14-fibroblasts, prior to co-injection with prostate tumor cells for the formation of xenograft

tumors in SCID mice, showed that the CXCL14-fibroblast-mediated increase in tumor growth and macrophage infiltration was dependent on NOS1. To answer the question if the NOS1-dependency remained across tumor types, the functions of CXCL14-fibroblasts were also investigated in a xenograft model of breast cancer. Co-injection of CXCL14-fibroblasts and MCF7 breast cancer cells significantly enhanced tumor growth, angiogenesis and macrophage infiltration, similar to the result obtained with the prostate tumor model. Downregulation of NOS1 in this model also reduced xenograft tumor growth and macrophage infiltration. Moreover, increased lymph angiogenesis was discovered as a novel protumoral function of CXCL14-expressing CAFs in both the breast and prostate tumor model.

In summary, these results show that the tumor promoting functions of CXCL14-fibroblasts are maintained by expression of NOS1. Targeting of the NOS1/NO signaling pathway in CXCL14-expressing CAF-subsets should be explored as a treatment intervention for breast and prostate cancer. Our study also encourages future exploration of the involvement of NOS1 in the downstream intracellular signaling of other chemokines.

6.2.2 Paper II

Expression of the chemokine CXCL14 in the tumor stroma is an independent marker of survival in breast cancer

CXCL14 is a chemokine with elevated expression in the tumor stroma of breast and prostate cancer. CXCL14 derived from cancer-associated fibroblasts have previously shown tumor promoting effects in mouse models of prostate and breast cancer. Earlier studies have demonstrated contradictory results on the role of CXCL14 in cancer progression and the clinical relevance of CXCL14. This could be explained by cell type-specific expression of CXCL14 or the unidentified receptor. In this study we investigated the clinical relevance and prognostic significance of compartment-specific expression of CXCL14 in a breast cancer cohort of 498 patients.

RNAscope analyses of CXCL14 mRNA expression revealed that breast cancer tissue display variable expression both in the tumor cells and in the tumor stroma. Associations of CXCL14 expression with clinicopathological parameters showed that epithelial CXCL14 expression was significantly associated with ERα positivity and low proliferation. On the contrary, stromal CXCL14 expression did not associate with any of the established clinicopathological parameters or subtypes of breast cancer. Notably, survival analysis identified CXCL14 expression in the tumor stroma as an independent marker of poor prognosis in breast cancer. High stromal, but not epithelial, levels of CXCL14 mRNA correlated significantly with shorter recurrence-free and breast cancer-specific survival in both uni- and multivariable analyses. Sub-set analyses showed that the correlation of stromal CXCL14 expression and worse clinical outcome was particularly prominent in patients of the ERα negative-, triple negative and basal subgroups, suggesting particular relevance for stromal CXCL14 in the progression of breast cancers belonging to these subgroups.

The finding of CXCL14 as a stromal prognostic marker adds to a number of recent studies identifying prognostic significance of stroma-expressed proteins, and stromal-gene-

signatures. Furthermore, novel clinical relevance of a stroma-derived secreted factor is here demonstrated. Based on the prognostic significance in difficult-to-treat subgroups of breast cancer, CXCL14 should also be considered as a candidate drug target. These results also encourage additional experimental studies to explore the mechanism behind the poor survival-associations, including paracrine effects of CXCL14 on tumor cells and studies on identification of a receptor for CXCL14.

6.2.3 Paper III

A novel ACKR2-dependent role of CAF-derived CXCL14 in epithelial-to-mesenchymal transition and metastasis of breast cancer

CAF-derived CXCL14 is an orphan chemokine that previously was shown to enhance prostate and breast tumor growth *in vivo* through NOS1 dependent mechanisms, and to associate with shorter survival of breast cancer patients. The underlying mechanisms of the poor-prognosis association of CXCL14 are not known. In this study we therefore explored the involvement of CAF-derived CXCL14 in tumor cell EMT, invasion and metastasis in tissue culture- and mice models and in patient gene expression datasets of breast cancer. To better understand the biological effects and tumor promoting activities of CXCL14 we also aimed to identify a receptor for the orphan chemokine.

CAF-derived CXCL14 was shown to promote formation of lung metastasis in SCID mice. Subsequent to tail-vein injection, metastasis was enhanced by "priming" of breast cancer cells *in vitro* with CXCL14-fibroblasts or by forced expression of CXCL14 in cancer cells. Mechanistic understanding of the pro-metastatic effects was provided by tissue culture- and xenograft models of breast cancer. Co-culture of breast cancer cells with CXCL14-fibroblast stimulated EMT, tumor cell migration and invasion. These could be directly mediated by recombinant CXCL14 but might also involve CXCL14 induced factors. The loss of epithelial markers and increase in mesenchymal markers and EMT transcription factors were also shown in a xenograft model of breast cancer where cancer cells were co-injected together with CXCL14-overexpressing fibroblasts.

In an effort to find a CXCL14-receptor, initial experiments were performed with pertussis toxin. These indicated that CXCL14, like other chemokines, signals through a member of the Gαi subfamily of GPCRs. Knock down of several CXCL14 receptor candidates demonstrated that the atypical G-protein coupled receptor (GPCR) ACKR2 mediated CXCL14-induced molecular signaling, including ERK phosphorylation and enhanced NOS1, and cellular responses, including fibroblast proliferation and breast cancer cell EMT, migration and invasion.

Clinical relevance of the experimental findings was demonstrated by correlations of the CXCL14 transcript with an EMT gene expression signature in different breast cancer gene expression data sets. The correlation was less prominent in patients of the Basal, HER2 and Luminal A subgroup with low ACKR2 expression, further supporting the role of ACKR2 as a novel CXCL14 receptor.

Altogether, these findings describe the first signaling receptor for CXCL14 and recognize previous unknown abilities of ACKRs to function as active transducers of chemokine

signaling. We also identify a novel clinically relevant role for CXCL14/ACKR2 signaling in inducing tumor cell EMT and invasion, important for formation of metastasis. The recent finding that stromal, but not epithelial expression, of CXCL14 is associated with shorter survival in breast cancer suggests that the paracrine stromal-epithelial CXCL14/ACKR2-signaling is most clinical relevant. Compartment-specific analyses of potential impact of ACKR2 have not been investigated but are prompted by the findings of the present study. Targeting of the CXCL14/ACKR2 pathway for treatment of breast cancer patients with a high stromal expression of CXCL14 should be explored in future studies. In addition, future studies should also consider the relevance of this pathway in other tumor types.

7 GENERAL OUTLOOK

Metastatic disease is the major cause of cancer death and there is no current treatment that efficiently cures patients with metastasis. Inhibition of the metastatic process poses an attractive strategy to improve cancer patient survival. For efficient inhibition of metastasis formation it is crucial to understand the underlying steps. Reactivation of developmental programs and the interplay between tumor cells and the microenvironment have been demonstrated to affect the metastatic cascade.

As described in this thesis, the reversible EMT/MET process -a program highly dependent on activation-signals from the tumor microenvironment- is relevant for the individual steps of metastasis formation. This developmental program constitutes a plausible target for treatment of metastatic disease. EMT might however be difficult to target due to the reversible process of MET that results in increased outgrowth of cancer cells at distant sites. One way to approach this problem is to identify EMT- and MET-mediators and to target these pathways individually or simultaneously. Studies on the regulators of EMT/MET are therefore highly warranted. In paper III we identified CXCL14/ACKR2 signaling to induce EMT, invasion and metastasis. Targeting EMT-inducing factors, such as CXCL14 or ACKR2, would be an option for metastasis prevention.

Also, tumor cells can remain dormant for years in recipient tissues before outgrowth is initiated. The ability of tumor cells to escape tumor dormancy depends partly on signals from the recipient tissue microenvironment. What are the microenvironmental factors that keep tumor cells in a dormant state, or alternatively activate them for proliferation and outgrowth? Since an inability of the EMT to MET transition can underlie dormancy, inhibition of stroma-produced inducers of MET appears as an interesting therapeutic opportunity.

To gain further mechanistic insight of which microenvironmental cues that allow only a minor number of cancer cells to undergo the different steps of metastasis and form secondary tumors is highly warranted. This thesis discusses the functions of chemokines, produced for example by stromal fibroblasts, in regulating metastasis formation. Chemokines enhance EMT and invasion of cancer cells in the primary tumor. In addition, expression of chemokines in the metastatic niche determines organotropism by directing spread of tumor cells to these organs. By interfering with certain chemokine-signaling pathways a dual targeting of metastasis formation could be obtained. Both dissemination of tumor cells, as a result of EMT and invasion, and the tissue colonization from early-disseminated tumor cells would be prevented. However, the involvement of CXCL14 in dictating organtropism to certain organs remains unknown. As reported in this thesis CXCL14 show high expression in brain, lungs and bone, which would suggest a preference for ACKR2 positive tumor cells to seed in these organs more efficiently than in other organs. Future studies should aim to explore this.

Besides effects of CXCL14 on tumor cells, the chemokine also plays a major role in enhancing CAF-tumor-promoting capabilities. In paper I we demonstrate that CXCL14-expressing CAFs promote tumor progression by upregulation of NOS1/NO signaling. The clinical importance for the role of CXCL14 in the stroma was demonstrated in paper II, and stromal expression was identified as a prognostic marker in breast cancer patients. The

studies in this thesis have revealed several candidate drug targets, whose targeting would interfere with the pro-tumoral effects of CAFs. These include CXCL14 itself, its receptor ACKR2 and components of the NOS1/NO signaling pathway.

Recent studies about transcriptional programs regulating CAF-phenotypes have shown the importance of EMT transcription factors, as discussed in this thesis. This indicates an involvement of EMT transcription factors -as well as other developmentally important transcription factors- in CAF-activation, and possibly determining different CAF-subsets. Thus, EMT-stimulating factors should be explored as mediators of CAF-heterogeneity. The identified function of CXCL14 in both enhancing tumor cell EMT and activating CAF-characteristics also supports this hypothesis, but further studies are needed.

The context-dependent pro- or anti-tumoral roles of CXCL14 during tumor progression should also be further analyzed to elucidate the underlying mechanism(s). We believe that cell type-specific expression of CXCL14 and its receptor could be one explanation. The identification of ACKR2 as a CXCL14-receptor will facilitate such studies and thereby significantly improve the possibilities of a more in-depth understanding of CXCL14 function in tumor biology.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer är ett samlingsnamn på flera sjukdomar som uppstår som en konsekvens av förändringar i våra gener. Dessa genförändringar bildas i flera steg över lång tid och har framförallt visat sig vara orsakade av livsstilsfaktorer. Det finns även ett fåtal genetiska förändringar som är nedärvda och när så är fallet talar man om ärftlig cancer. Vilken typ av cancer man drabbas av beror på i vilken vävnad och celltyp som de genetiska förändringarna har uppstått. Bröst- och prostata cancer hör till de vanligaste cancerformerna. 9000 kvinnor drabbas årligen av bröstcancer i Sverige och ungefär lika många män drabbas av prostatacancer.

Normalt bildas det kontinuerligt nya celler i alla kroppens vävnader genom kontrollerade celldelningar. När en cell har erhållit genetiska förändringar stängs dessa kontrollprogram av och celldelningen kommer i obalans. Gener som driver på tillväxt får en ökad aktivitet medan gener som bromsar tillväxt får en minskad. Trots att cancer består av många olika sjukdomar har cancerceller från olika cancerformer vissa gemensamma karaktärsdrag. De producerar sina egna tillväxtfaktorer och är okänsliga mot tillväxthämmande signaler. De har motståndskraft mot att dö och istället en förmåga att dela sig i oändlighet. Cellerna växer på varandra och bildar så småningom en klump, en tumör. Denna tumör fortsätter att växa och cancerceller stimulerar blodkärlsbildning som gör att tumören förses med syre och tillväxtfaktorer, så att den kan växa sig ännu större. Ytterligare ett karaktärsdrag är att cancerceller har en ökad förmåga att flytta på sig och invadera in i omkringliggande vävnader och in i blod- eller lymfkärl. Väl inne i blodet färdas de genom kroppen och kan ta sig vidare in i nya organ, där de kan bilda tumörer på nytt, så kallade dottertumörer eller metastaser.

Tumörer består inte enbart av cancerceller utan även av andra celltyper och molekyler, det så kallade tumörstromat, som kommunicerar med cancercellerna för att stimulera tumörtillväxt ytterligare. En av de vanligaste celltyperna i stromat är cancer-associerade fibroblaster som påverkar tumörens tillväxt, utveckling och metastasering genom ett samspel med cancerceller och andra stromala celler. Detta samspel äger rum genom utsöndring av signalmolekyler, vilka binder till specifika målmolekyler, receptorer, på mottagarcellerna.

En sådan signalmolekyl är CXCL14 som produceras av fibroblaster i tumörstromat i bröst och prostatacancer. Fibroblast-deriverat CXCL14 är viktigt för tillväxt av både bröst- och prostatacancerceller i experimentella modeller. CXCL14 kan även förändra tumörcellerna så att de blir mer migratoriska och invarderar närliggande vävnader, samt ökar metastasering. Den receptor som CXCL14 binder till på cancercellerna har tidigare varit okänd. Vi har identifierat en receptor, ACKR2, som är involverad i CXCL14s biologiska effekter. Genom att experimentellt ”ta bort” denna receptor minskas cancercellernas intracellulära signaliering, migration och invasion. Vi har även lyckats identifiera en annan tumörstimulatiorisk molekyl, NOS1 som induceras av CXCL14. I prover från bröstcancerpatienter har vi också visat att stromalt CXCL14 är kopplat till sämre överlevnad och stromalt CXCL14 identifierades som en prognostisk markör. Genom att antingen utveckla läkemedel riktade mot CXCL14 själv, dess receptor ACKR2 eller NOS1-signalering tror vi att utvecklingen av prostata- och bröstcancer skulle kunna hämmas. Det behövs emellertid prövas i framtida studier om så är fallet.

9 ACKNOWLEDGEMENTS

I would like to start by thanking my main supervisor **Arne Östman.** The support, encouragement and freedom you give have meant a lot to me during these years. You are a true role model for the research community and I believe everyone has something to learn from you. Also, thank you for being a very warm and understanding person. It has been absolutely fantastic to work for you and I will try not to be too sad that it's over soon.

My co-supervisor **Martin Augsten**. Thank you for your enthusiasm and hours of fun research discussions and experiment planning. I think you are the only one, except me, who truly enjoys talking about the contradictory role of CXCL14 in tumor biology ☺. Thank you for a great teamwork over the years, both in the lab and also on the football field.

I would also like to thank past and present members of the Östman lab: Thank you for endless love and support!

Janna, I always told you: "I don't know how I will manage without you in the lab". I managed ☺, but without anyone to run, go to "cirkelfys", eating "knäckebröd", drinking to much wine and gossiping with. Thank you for all that and so much more! It has been very empty the last year.

Linda, thank you for EVERYTHING! You are everything a good friend should be: always encouraging, great humor and the best listener. The Elin-show and Harald would never ever have existed without you. You always make me happy and I so look forward seeing you more often. Still miss you like crazy.

Carina, thank you for all the fun times. Without you I would never have tried pole-dance, walking in real ballet shoes, hitting people with sticks (sword) at kendo, or eating strange Belarusian candy. Also, thank you for sad times. Besides, being a very good friend I truly admire your scientific skills and hard work. If you don't succeed as a researcher, no one else deserves to.

Åsa, thank you for always being nice and helpful! I have missed your strange, but extremely funny humor. Thank you for all the memorable moments including, African dance at Friskis, "kräftskiva" at your place and all the delicious cakes you made.

Laura, thank you for a wonderful fun and productive year working together. Keep on being positive, it is contagious and we all need more of it. Also, thank you for all the German sausages, all the "te-fikas" and runs we shared. And Laura, now I have "my own Kaplan-Meier curve".

Alessandro, thank you for being the best singing-partner in the cell lab, for dragging me to the gym and for all the fun times gossiping and dancing.

Magnus, it is always as fun hanging out with you. I took a while but I hope you have learned after these years when I tell a joke and when I tell the truth. Thank you for all great laughs.

And who knows, if you finish your PhD-studies soon, we might become colleagues instead of you being personnel ☺.

Thank you: **Monika**, for valuable comments and input, and fantastic chocolate cakes. **Sara C**, for entertaining stories and for providing the best "lakritssnaps" I have ever tried. **Artur**, for your invaluable SPSS-knowledge and listening to me complaining in the office. **Jeroen**, thanks for just being brilliant, Mega-mindy sends greetings. **Markus**, for all the fun story-telling and your inspiring interest for every detail in an experiment. **Mercedes**, your happy face always make me smile. **Christina**, what happened to morning coffee when you left? Thanks to all other members of the lab over the years. Special thanks to: **Hanna, Maja, Patrik, Helene, Sara M, Chern, Cristina and Jai.**

I would also like to thank all collaborative partners, especially **John Lövrot**. Thank you for all the analyses and your patience.

To all past and **present colleagues and friends** at CCK, thank you a lot! A special thanks to Monika Nister group for letting me in on the fifth floor. Also, a big thank you to all the players in "CCK champions".

To my friends and family:

Marcus, for being a great host and for introducing me into the "octoberfest-life style"!

Sofia, for all the fun we have shared both outside and at CCK. Som en gummiboll…☺

SAKEFELE. Tack för allt skratt och gråt under åren. Bättre vänner finns inte.

Bengt, Else-Marie och **Joel**, tack för att ni har givit mig ett sådant varmt välkomnande in i Hägerstrand-familjen. **Joel**, jag ser fram emot många fler spelkvällar och femkamper. ☺

Mamma och **Pappa**, tack för att ni alltid har stöttat mig och låtit mig göra exakt det jag vill. Jag gör det jag tycker bäst om och det är så mycket tack vare er. **Linus** och **Matts**, tack för att ni är de bästa storebröderna en lillasyster kan ha.

And at last.

I have always said that I can't stand a cute acknowledgement, so I will try my best just to be honest. **Daniel**. This thesis would have been possible without you. But I have to say, to have proper dinners on clean plates has been so much nicer than just eating yogurt on dirty ones. Thank you for always challenging me, I need that. You have such a brilliant mind and the biggest heart. I know you hate my curiosity, but honestly… curiosity never killed the cat, it just made it more curious! Tack för att du är lika knäpp som jag. Jag älskar dig!

10 REFERENCES

1. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
2. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
3. Sporn, M.B. The war on cancer. *Lancet* **347**, 1377-1381 (1996).
4. Paget, S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer metastasis reviews* **8**, 98-101 (1989).
5. Langley, R.R. & Fidler, I.J. The seed and soil hypothesis revisited--the role of tumor-stroma interactions in metastasis to different organs. *International journal of cancer. Journal international du cancer* **128**, 2527-2535 (2011).
6. Valastyan, S. & Weinberg, R.A. Tumor metastasis: molecular insights and evolving paradigms. *Cell* **147**, 275-292 (2011).
7. Bos, P.D.*, et al.* Genes that mediate breast cancer metastasis to the brain. *Nature* **459**, 1005-1009 (2009).
8. Minn, A.J.*, et al.* Genes that mediate breast cancer metastasis to lung. *Nature* **436**, 518-524 (2005).
9. Kang, Y.*, et al.* A multigenic program mediating breast cancer metastasis to bone. *Cancer cell* **3**, 537-549 (2003).
10. Tabaries, S.*, et al.* Claudin-2 is selectively enriched in and promotes the formation of breast cancer liver metastases through engagement of integrin complexes. *Oncogene* **30**, 1318-1328 (2011).
11. Joyce, J.A. & Pollard, J.W. Microenvironmental regulation of metastasis. *Nature reviews. Cancer* **9**, 239-252 (2009).
12. Fidler, I.J. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nature reviews. Cancer* **3**, 453-458 (2003).
13. Kalluri, R. & Weinberg, R.A. The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation* **119**, 1420-1428 (2009).
14. Steeg, P.S. Tumor metastasis: mechanistic insights and clinical challenges. *Nature medicine* **12**, 895-904 (2006).
15. Kaplan, R.N.*, et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438**, 820-827 (2005).
16. McAllister, S.S.*, et al.* Systemic endocrine instigation of indolent tumor growth requires osteopontin. *Cell* **133**, 994-1005 (2008).
17. Psaila, B. & Lyden, D. The metastatic niche: adapting the foreign soil. *Nature reviews. Cancer* **9**, 285-293 (2009).
18. Erler, J.T.*, et al.* Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer cell* **15**, 35-44 (2009).
19. Cox, T.R.*, et al.* The hypoxic cancer secretome induces pre-metastatic bone lesions through lysyl oxidase. *Nature* **522**, 106-110 (2015).
20. Hoshino, A.*, et al.* Tumour exosome integrins determine organotropic metastasis. *Nature* **527**, 329-335 (2015).
21. Bruzzese, F.*, et al.* Local and systemic protumorigenic effects of cancer-associated fibroblast-derived GDF15. *Cancer research* **74**, 3408-3417 (2014).
22. Thiery, J.P., Acloque, H., Huang, R.Y. & Nieto, M.A. Epithelial-mesenchymal transitions in development and disease. *Cell* **139**, 871-890 (2009).
23. Thiery, J.P. Epithelial-mesenchymal transitions in tumour progression. *Nature reviews. Cancer* **2**, 442-454 (2002).
24. Yilmaz, M. & Christofori, G. EMT, the cytoskeleton, and cancer cell invasion. *Cancer metastasis reviews* **28**, 15-33 (2009).

25. Ostman, A. & Augsten, M. Cancer-associated fibroblasts and tumor growth--bystanders turning into key players. *Current opinion in genetics & development* **19**, 67-73 (2009).
26. Fischer, K.R.*, et al.* Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. *Nature* **527**, 472-476 (2015).
27. Zheng, X.*, et al.* Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. *Nature* **527**, 525-530 (2015).
28. Puisieux, A., Brabletz, T. & Caramel, J. Oncogenic roles of EMT-inducing transcription factors. *Nature cell biology* **16**, 488-494 (2014).
29. Droufakou, S.*, et al.* Multiple ways of silencing E-cadherin gene expression in lobular carcinoma of the breast. *International journal of cancer. Journal international du cancer* **92**, 404-408 (2001).
30. Onder, T.T.*, et al.* Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer research* **68**, 3645-3654 (2008).
31. Christofori, G. & Semb, H. The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends in biochemical sciences* **24**, 73-76 (1999).
32. Perl, A.K., Wilgenbus, P., Dahl, U., Semb, H. & Christofori, G. A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* **392**, 190-193 (1998).
33. Olmeda, D., Jorda, M., Peinado, H., Fabra, A. & Cano, A. Snail silencing effectively suppresses tumour growth and invasiveness. *Oncogene* **26**, 1862-1874 (2007).
34. Ota, I., Li, X.Y., Hu, Y. & Weiss, S.J. Induction of a MT1-MMP and MT2-MMP-dependent basement membrane transmigration program in cancer cells by Snail1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 20318-20323 (2009).
35. Shih, J.Y.*, et al.* Transcription repressor slug promotes carcinoma invasion and predicts outcome of patients with lung adenocarcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 8070-8078 (2005).
36. Kessenbrock, K., Plaks, V. & Werb, Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* **141**, 52-67 (2010).
37. Egeblad, M. & Werb, Z. New functions for the matrix metalloproteinases in cancer progression. *Nature reviews. Cancer* **2**, 161-174 (2002).
38. Kim, S.A.*, et al.* Loss of CDH1 (E-cadherin) expression is associated with infiltrative tumour growth and lymph node metastasis. *British journal of cancer* **114**, 199-206 (2016).
39. Hirohashi, S. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *The American journal of pathology* **153**, 333-339 (1998).
40. Prat, A.*, et al.* Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast cancer research : BCR* **12**, R68 (2010).
41. Sarrio, D.*, et al.* Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer research* **68**, 989-997 (2008).
42. Sorlie, T.*, et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 10869-10874 (2001).
43. Livasy, C.A.*, et al.* Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **19**, 264-271 (2006).
44. Vincent, T.*, et al.* A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-beta mediated epithelial-mesenchymal transition. *Nature cell biology* **11**, 943-950 (2009).

45. Brabletz, T.*, et al.* Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 10356-10361 (2001).
46. Brabletz, T.*, et al.* Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. *Cells, tissues, organs* **179**, 56-65 (2005).
47. Celesti, G.*, et al.* Presence of Twist1-positive neoplastic cells in the stroma of chromosome-unstable colorectal tumors. *Gastroenterology* **145**, 647-657 e615 (2013).
48. Drake, J.M., Strohbehn, G., Bair, T.B., Moreland, J.G. & Henry, M.D. ZEB1 enhances transendothelial migration and represses the epithelial phenotype of prostate cancer cells. *Molecular biology of the cell* **20**, 2207-2217 (2009).
49. Tsuji, T.*, et al.* Epithelial-mesenchymal transition induced by growth suppressor p12CDK2-AP1 promotes tumor cell local invasion but suppresses distant colony growth. *Cancer research* **68**, 10377-10386 (2008).
50. Muraoka, R.S.*, et al.* Blockade of TGF-beta inhibits mammary tumor cell viability, migration, and metastases. *The Journal of clinical investigation* **109**, 1551-1559 (2002).
51. Yu, M.*, et al.* Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* **339**, 580-584 (2013).
52. Tsai, J.H., Donaher, J.L., Murphy, D.A., Chau, S. & Yang, J. Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. *Cancer cell* **22**, 725-736 (2012).
53. Hou, J.M.*, et al.* Circulating tumor cells as a window on metastasis biology in lung cancer. *The American journal of pathology* **178**, 989-996 (2011).
54. Kallergi, G.*, et al.* Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. *Breast cancer research : BCR* **13**, R59 (2011).
55. Min, A.L.*, et al.* High expression of Snail mRNA in blood from hepatocellular carcinoma patients with extra-hepatic metastasis. *Clinical & experimental metastasis* **26**, 759-767 (2009).
56. Rhim, A.D.*, et al.* EMT and dissemination precede pancreatic tumor formation. *Cell* **148**, 349-361 (2012).
57. Husemann, Y.*, et al.* Systemic spread is an early step in breast cancer. *Cancer cell* **13**, 58-68 (2008).
58. Bonnomet, A.*, et al.* A dynamic in vivo model of epithelial-to-mesenchymal transitions in circulating tumor cells and metastases of breast cancer. *Oncogene* **31**, 3741-3753 (2012).
59. Derksen, P.W.*, et al.* Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer cell* **10**, 437-449 (2006).
60. Labelle, M., Begum, S. & Hynes, R.O. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer cell* **20**, 576-590 (2011).
61. Cohen, S.J.*, et al.* Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **26**, 3213-3221 (2008).
62. Stoletov, K.*, et al.* Visualizing extravasation dynamics of metastatic tumor cells. *Journal of cell science* **123**, 2332-2341 (2010).

63. Celia-Terrassa, T.*, et al.* Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. *The Journal of clinical investigation* **122**, 1849-1868 (2012).
64. Chao, Y.L., Shepard, C.R. & Wells, A. Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. *Molecular cancer* **9**, 179 (2010).
65. Vega, S.*, et al.* Snail blocks the cell cycle and confers resistance to cell death. *Genes & development* **18**, 1131-1143 (2004).
66. Mejlvang, J.*, et al.* Direct repression of cyclin D1 by SIP1 attenuates cell cycle progression in cells undergoing an epithelial mesenchymal transition. *Molecular biology of the cell* **18**, 4615-4624 (2007).
67. Evdokimova, V.*, et al.* Translational activation of snail1 and other developmentally regulated transcription factors by YB-1 promotes an epithelial-mesenchymal transition. *Cancer cell* **15**, 402-415 (2009).
68. Ocana, O.H.*, et al.* Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer Prrx1. *Cancer cell* **22**, 709-724 (2012).
69. Gao, D.*, et al.* Myeloid progenitor cells in the premetastatic lung promote metastases by inducing mesenchymal to epithelial transition. *Cancer research* **72**, 1384-1394 (2012).
70. Del Pozo Martin, Y.*, et al.* Mesenchymal Cancer Cell-Stroma Crosstalk Promotes Niche Activation, Epithelial Reversion, and Metastatic Colonization. *Cell reports* **13**, 2456-2469 (2015).
71. Pietras, K. & Ostman, A. Hallmarks of cancer: interactions with the tumor stroma. *Experimental cell research* **316**, 1324-1331 (2010).
72. Hynes, R.O. The extracellular matrix: not just pretty fibrils. *Science* **326**, 1216-1219 (2009).
73. Butcher, D.T., Alliston, T. & Weaver, V.M. A tense situation: forcing tumour progression. *Nature reviews. Cancer* **9**, 108-122 (2009).
74. Levental, K.R.*, et al.* Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell* **139**, 891-906 (2009).
75. Hasebe, T.*, et al.* Prognostic significance of fibrotic focus in invasive ductal carcinoma of the breast: a prospective observational study. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **15**, 502-516 (2002).
76. Conklin, M.W.*, et al.* Aligned collagen is a prognostic signature for survival in human breast carcinoma. *The American journal of pathology* **178**, 1221-1232 (2011).
77. Wei, S.C.*, et al.* Matrix stiffness drives epithelial-mesenchymal transition and tumour metastasis through a TWIST1-G3BP2 mechanotransduction pathway. *Nature cell biology* **17**, 678-688 (2015).
78. Vellinga, T.T.*, et al.* Collagen-rich stroma in aggressive colon tumors induces mesenchymal gene expression and tumor cell invasion. *Oncogene* (2016).
79. Zhang, K.*, et al.* The collagen receptor discoidin domain receptor 2 stabilizes SNAIL1 to facilitate breast cancer metastasis. *Nature cell biology* **15**, 677-687 (2013).
80. Erler, J.T.*, et al.* Lysyl oxidase is essential for hypoxia-induced metastasis. *Nature* **440**, 1222-1226 (2006).
81. El-Haibi, C.P.*, et al.* Critical role for lysyl oxidase in mesenchymal stem cell-driven breast cancer malignancy. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 17460-17465 (2012).
82. Oskarsson, T.*, et al.* Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nature medicine* **17**, 867-874 (2011).
83. Malanchi, I.*, et al.* Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature* **481**, 85-89 (2012).

84. O'Connell, J.T.*, et al.* VEGF-A and Tenascin-C produced by S100A4+ stromal cells are important for metastatic colonization. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 16002-16007 (2011).
85. Tian, X.*, et al.* High-molecular-mass hyaluronan mediates the cancer resistance of the naked mole rat. *Nature* **499**, 346-349 (2013).
86. Folkman, J. & Hanahan, D. Switch to the angiogenic phenotype during tumorigenesis. *Princess Takamatsu symposia* **22**, 339-347 (1991).
87. Weis, S.M. & Cheresh, D.A. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nature medicine* **17**, 1359-1370 (2011).
88. Bergers, G. & Benjamin, L.E. Tumorigenesis and the angiogenic switch. *Nature reviews. Cancer* **3**, 401-410 (2003).
89. Baluk, P., Hashizume, H. & McDonald, D.M. Cellular abnormalities of blood vessels as targets in cancer. *Current opinion in genetics & development* **15**, 102-111 (2005).
90. Paez-Ribes, M.*, et al.* Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer cell* **15**, 220-231 (2009).
91. Ebos, J.M.*, et al.* Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer cell* **15**, 232-239 (2009).
92. Branco-Price, C.*, et al.* Endothelial cell HIF-1alpha and HIF-2alpha differentially regulate metastatic success. *Cancer cell* **21**, 52-65 (2012).
93. Wolf, M.J.*, et al.* Endothelial CCR2 signaling induced by colon carcinoma cells enables extravasation via the JAK2-Stat5 and p38MAPK pathway. *Cancer cell* **22**, 91-105 (2012).
94. Ghajar, C.M.*, et al.* The perivascular niche regulates breast tumour dormancy. *Nature cell biology* **15**, 807-817 (2013).
95. Stacker, S.A.*, et al.* Lymphangiogenesis and lymphatic vessel remodelling in cancer. *Nature reviews. Cancer* **14**, 159-172 (2014).
96. Stacker, S.A.*, et al.* VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nature medicine* **7**, 186-191 (2001).
97. Skobe, M.*, et al.* Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nature medicine* **7**, 192-198 (2001).
98. Dadras, S.S.*, et al.* Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. *The American journal of pathology* **162**, 1951-1960 (2003).
99. Doeden, K.*, et al.* Lymphatic invasion in cutaneous melanoma is associated with sentinel lymph node metastasis. *Journal of cutaneous pathology* **36**, 772-780 (2009).
100. Bono, P.*, et al.* High LYVE-1-positive lymphatic vessel numbers are associated with poor outcome in breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10**, 7144-7149 (2004).
101. Saad, R.S.*, et al.* Lymphatic microvessel density as prognostic marker in colorectal cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **19**, 1317-1323 (2006).
102. Armulik, A., Genove, G. & Betsholtz, C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Developmental cell* **21**, 193-215 (2011).
103. Betsholtz, C., Lindblom, P. & Gerhardt, H. Role of pericytes in vascular morphogenesis. *Exs*, 115-125 (2005).
104. Furuhashi, M.*, et al.* Platelet-derived growth factor production by B16 melanoma cells leads to increased pericyte abundance in tumors and an associated increase in tumor growth rate. *Cancer research* **64**, 2725-2733 (2004).
105. Armulik, A., Abramsson, A. & Betsholtz, C. Endothelial/pericyte interactions. *Circulation research* **97**, 512-523 (2005).

106. Cooke, V.G.*, et al.* Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. *Cancer cell* **21**, 66-81 (2012).
107. Xian, X.*, et al.* Pericytes limit tumor cell metastasis. *The Journal of clinical investigation* **116**, 642-651 (2006).
108. O'Keeffe, M.B.*, et al.* Investigation of pericytes, hypoxia, and vascularity in bladder tumors: association with clinical outcomes. *Oncology research* **17**, 93-101 (2008).
109. Stefansson, I.M., Salvesen, H.B. & Akslen, L.A. Vascular proliferation is important for clinical progress of endometrial cancer. *Cancer research* **66**, 3303-3309 (2006).
110. Yonenaga, Y.*, et al.* Absence of smooth muscle actin-positive pericyte coverage of tumor vessels correlates with hematogenous metastasis and prognosis of colorectal cancer patients. *Oncology* **69**, 159-166 (2005).
111. Qian, C.N., Huang, D., Wondergem, B. & Teh, B.T. Complexity of tumor vasculature in clear cell renal cell carcinoma. *Cancer* **115**, 2282-2289 (2009).
112. Morikawa, S.*, et al.* Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *The American journal of pathology* **160**, 985-1000 (2002).
113. Song, S., Ewald, A.J., Stallcup, W., Werb, Z. & Bergers, G. PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nature cell biology* **7**, 870-879 (2005).
114. Dulauroy, S., Di Carlo, S.E., Langa, F., Eberl, G. & Peduto, L. Lineage tracing and genetic ablation of ADAM12(+) perivascular cells identify a major source of profibrotic cells during acute tissue injury. *Nature medicine* **18**, 1262-1270 (2012).
115. Corvigno, S.*, et al.* Markers of fibroblast-rich tumor stroma and perivascular cells in serous ovarian cancer: inter- and intra-patient heterogeneity and impact on survival. *Oncotarget* (2016).
116. Hosaka, K.*, et al.* Tumour PDGF-BB expression levels determine dual effects of anti-PDGF drugs on vascular remodelling and metastasis. *Nature communications* **4**, 2129 (2013).
117. Bergers, G., Song, S., Meyer-Morse, N., Bergsland, E. & Hanahan, D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *The Journal of clinical investigation* **111**, 1287-1295 (2003).
118. Erber, R.*, et al.* Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **18**, 338-340 (2004).
119. Franco, M., Roswall, P., Cortez, E., Hanahan, D. & Pietras, K. Pericytes promote endothelial cell survival through induction of autocrine VEGF-A signaling and Bcl-w expression. *Blood* **118**, 2906-2917 (2011).
120. Keskin, D.*, et al.* Targeting vascular pericytes in hypoxic tumors increases lung metastasis via angiopoietin-2. *Cell reports* **10**, 1066-1081 (2015).
121. Nash, G.F., Turner, L.F., Scully, M.F. & Kakkar, A.K. Platelets and cancer. *The lancet oncology* **3**, 425-430 (2002).
122. Labelle, M., Begum, S. & Hynes, R.O. Platelets guide the formation of early metastatic niches. *Proceedings of the National Academy of Sciences of the United States of America* **111**, E3053-3061 (2014).
123. Schumacher, D., Strilic, B., Sivaraj, K.K., Wettschureck, N. & Offermanns, S. Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. *Cancer cell* **24**, 130-137 (2013).
124. Coupland, L.A., Chong, B.H. & Parish, C.R. Platelets and P-selectin control tumor cell metastasis in an organ-specific manner and independently of NK cells. *Cancer research* **72**, 4662-4671 (2012).

125. Rothwell, P.M.*, et al.* Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet* **379**, 1591-1601 (2012).
126. Talmadge, J.E., Donkor, M. & Scholar, E. Inflammatory cell infiltration of tumors: Jekyll or Hyde. *Cancer metastasis reviews* **26**, 373-400 (2007).
127. Khalil, D.N., Smith, E.L., Brentjens, R.J. & Wolchok, J.D. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nature reviews. Clinical oncology* (2016).
128. Mahoney, K.M., Rennert, P.D. & Freeman, G.J. Combination cancer immunotherapy and new immunomodulatory targets. *Nature reviews. Drug discovery* **14**, 561-584 (2015).
129. de Visser, K.E., Eichten, A. & Coussens, L.M. Paradoxical roles of the immune system during cancer development. *Nature reviews. Cancer* **6**, 24-37 (2006).
130. DeNardo, D.G., Johansson, M. & Coussens, L.M. Immune cells as mediators of solid tumor metastasis. *Cancer metastasis reviews* **27**, 11-18 (2008).
131. Azab, B.*, et al.* Usefulness of the neutrophil-to-lymphocyte ratio in predicting short- and long-term mortality in breast cancer patients. *Annals of surgical oncology* **19**, 217-224 (2012).
132. Noh, H., Eomm, M. & Han, A. Usefulness of pretreatment neutrophil to lymphocyte ratio in predicting disease-specific survival in breast cancer patients. *Journal of breast cancer* **16**, 55-59 (2013).
133. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in immunology* **23**, 549-555 (2002).
134. Pollard, J.W. Tumour-educated macrophages promote tumour progression and metastasis. *Nature reviews. Cancer* **4**, 71-78 (2004).
135. Campbell, M.J.*, et al.* Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome. *Breast cancer research and treatment* **128**, 703-711 (2011).
136. Bingle, L., Brown, N.J. & Lewis, C.E. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *The Journal of pathology* **196**, 254-265 (2002).
137. Leek, R.D.*, et al.* Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer research* **56**, 4625-4629 (1996).
138. Lewis, C.E. & Pollard, J.W. Distinct role of macrophages in different tumor microenvironments. *Cancer research* **66**, 605-612 (2006).
139. Farinha, P.*, et al.* Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL). *Blood* **106**, 2169-2174 (2005).
140. Varney, M.L., Johansson, S.L. & Singh, R.K. Tumour-associated macrophage infiltration, neovascularization and aggressiveness in malignant melanoma: role of monocyte chemotactic protein-1 and vascular endothelial growth factor-A. *Melanoma research* **15**, 417-425 (2005).
141. Forssell, J.*, et al.* High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 1472-1479 (2007).
142. Ong, S.M.*, et al.* Macrophages in human colorectal cancer are pro-inflammatory and prime T cells towards an anti-tumour type-1 inflammatory response. *European journal of immunology* **42**, 89-100 (2012).
143. Mosser, D.M. & Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology* **8**, 958-969 (2008).

144. Coffelt, S.B., Hughes, R. & Lewis, C.E. Tumor-associated macrophages: effectors of angiogenesis and tumor progression. *Biochimica et biophysica acta* **1796**, 11-18 (2009).
145. Hao, N.B.*, et al.* Macrophages in tumor microenvironments and the progression of tumors. *Clinical & developmental immunology* **2012**, 948098 (2012).
146. Kitamura, T.*, et al.* Inactivation of chemokine (C-C motif) receptor 1 (CCR1) suppresses colon cancer liver metastasis by blocking accumulation of immature myeloid cells in a mouse model. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 13063-13068 (2010).
147. Qian, B.Z.*, et al.* CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* **475**, 222-225 (2011).
148. Mantovani, A. & Sica, A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Current opinion in immunology* **22**, 231-237 (2010).
149. Franklin, R.A.*, et al.* The cellular and molecular origin of tumor-associated macrophages. *Science* **344**, 921-925 (2014).
150. Xue, J.*, et al.* Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* **40**, 274-288 (2014).
151. Lin, E.Y., Nguyen, A.V., Russell, R.G. & Pollard, J.W. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *The Journal of experimental medicine* **193**, 727-740 (2001).
152. Hiratsuka, S.*, et al.* MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer cell* **2**, 289-300 (2002).
153. DeNardo, D.G.*, et al.* CD4(+) T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer cell* **16**, 91-102 (2009).
154. Zhang, J.*, et al.* Regulation of epithelial-mesenchymal transition by tumor-associated macrophages in cancer. *American journal of translational research* **7**, 1699-1711 (2015).
155. Su, S.*, et al.* A positive feedback loop between mesenchymal-like cancer cells and macrophages is essential to breast cancer metastasis. *Cancer cell* **25**, 605-620 (2014).
156. Yang, L.*, et al.* Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. *Cancer cell* **13**, 23-35 (2008).
157. Nielsen, S.R.*, et al.* Macrophage-secreted granulin supports pancreatic cancer metastasis by inducing liver fibrosis. *Nature cell biology* **18**, 549-560 (2016).
158. Liang, W. & Ferrara, N. The Complex Role of Neutrophils in Tumor Angiogenesis and Metastasis. *Cancer immunology research* **4**, 83-91 (2016).
159. Wculek, S.K. & Malanchi, I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature* **528**, 413-417 (2015).
160. Coffelt, S.B.*, et al.* IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* **522**, 345-348 (2015).
161. Granot, Z.*, et al.* Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer cell* **20**, 300-314 (2011).
162. Vermeulen, L.*, et al.* Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. *Nature cell biology* **12**, 468-476 (2010).
163. Kraman, M.*, et al.* Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science* **330**, 827-830 (2010).
164. Valencia, T.*, et al.* Metabolic reprogramming of stromal fibroblasts through p62-mTORC1 signaling promotes inflammation and tumorigenesis. *Cancer cell* **26**, 121-135 (2014).
165. Martinez-Outschoorn, U.E., Sotgia, F. & Lisanti, M.P. Metabolic asymmetry in cancer: a "balancing act" that promotes tumor growth. *Cancer cell* **26**, 5-7 (2014).

166. Kalluri, R. & Zeisberg, M. Fibroblasts in cancer. *Nature reviews. Cancer* **6**, 392-401 (2006).
167. Dvorak, H.F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *The New England journal of medicine* **315**, 1650-1659 (1986).
168. Augsten, M. Cancer-associated fibroblasts as another polarized cell type of the tumor microenvironment. *Frontiers in oncology* **4**, 62 (2014).
169. Direkze, N.C.*, et al.* Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer research* **64**, 8492-8495 (2004).
170. Ishii, G.*, et al.* Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. *Biochemical and biophysical research communications* **309**, 232-240 (2003).
171. Direkze, N.C.*, et al.* Bone marrow-derived stromal cells express lineage-related messenger RNA species. *Cancer research* **66**, 1265-1269 (2006).
172. Mishra, P.J.*, et al.* Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. *Cancer research* **68**, 4331-4339 (2008).
173. Kim, K.K.*, et al.* Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 13180-13185 (2006).
174. Radisky, D.C., Kenny, P.A. & Bissell, M.J. Fibrosis and cancer: do myofibroblasts come also from epithelial cells via EMT? *Journal of cellular biochemistry* **101**, 830-839 (2007).
175. Iwano, M.*, et al.* Evidence that fibroblasts derive from epithelium during tissue fibrosis. *The Journal of clinical investigation* **110**, 341-350 (2002).
176. Zeisberg, M.*, et al.* BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. *Nature medicine* **9**, 964-968 (2003).
177. Zeisberg, E.M., Potenta, S., Xie, L., Zeisberg, M. & Kalluri, R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer research* **67**, 10123-10128 (2007).
178. Dulauroy, S., Di Carlo, S.E., Langa, F., Eberl, G. & Peduto, L. Lineage tracing and genetic ablation of ADAM12(+) perivascular cells identify a major source of profibrotic cells during acute tissue injury. *Nature medicine* (2012).
179. Sugimoto, H., Mundel, T.M., Kieran, M.W. & Kalluri, R. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer biology & therapy* **5**, 1640-1646 (2006).
180. Scherz-Shouval, R.*, et al.* The reprogramming of tumor stroma by HSF1 is a potent enabler of malignancy. *Cell* **158**, 564-578 (2014).
181. Stanisavljevic, J.*, et al.* Snail1-expressing fibroblasts in the tumor microenvironment display mechanical properties that support metastasis. *Cancer research* **75**, 284-295 (2015).
182. Garcia-Palmero, I.*, et al.* Twist1-induced activation of human fibroblasts promotes matrix stiffness by upregulating palladin and collagen alpha1(VI). *Oncogene* (2016).
183. Lee, K.W., Yeo, S.Y., Sung, C.O. & Kim, S.H. Twist1 is a key regulator of cancer-associated fibroblasts. *Cancer research* **75**, 73-85 (2015).
184. Sung, C.O., Lee, K.W., Han, S. & Kim, S.H. Twist1 is up-regulated in gastric cancer-associated fibroblasts with poor clinical outcomes. *The American journal of pathology* **179**, 1827-1838 (2011).
185. Orr, B.*, et al.* Identification of stromally expressed molecules in the prostate by tag-profiling of cancer-associated fibroblasts, normal fibroblasts and fetal prostate. *Oncogene* **31**, 1130-1142 (2012).

186. Saito, R.A., *et al.* Forkhead box F1 regulates tumor-promoting properties of cancer-associated fibroblasts in lung cancer. *Cancer research* **70**, 2644-2654 (2010).
187. Calvo, F., *et al.* Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts. *Nature cell biology* **15**, 637-646 (2013).
188. Sherman, M.H., *et al.* Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. *Cell* **159**, 80-93 (2014).
189. Albrengues, J., *et al.* Epigenetic switch drives the conversion of fibroblasts into proinvasive cancer-associated fibroblasts. *Nature communications* **6**, 10204 (2015).
190. Flaberg, E., *et al.* High-throughput live-cell imaging reveals differential inhibition of tumor cell proliferation by human fibroblasts. *International journal of cancer. Journal international du cancer* **128**, 2793-2802 (2011).
191. Alkasalias, T., *et al.* Inhibition of tumor cell proliferation and motility by fibroblasts is both contact and soluble factor dependent. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 17188-17193 (2014).
192. Wadlow, R.C., *et al.* Systems-level modeling of cancer-fibroblast interaction. *PloS one* **4**, e6888 (2009).
193. Rhim, A.D., *et al.* Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. *Cancer cell* **25**, 735-747 (2014).
194. Shin, K., *et al.* Hedgehog signaling restrains bladder cancer progression by eliciting stromal production of urothelial differentiation factors. *Cancer cell* **26**, 521-533 (2014).
195. Ozdemir, B.C., *et al.* Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. *Cancer cell* **25**, 719-734 (2014).
196. Bhowmick, N.A., Neilson, E.G. & Moses, H.L. Stromal fibroblasts in cancer initiation and progression. *Nature* **432**, 332-337 (2004).
197. Orimo, A., *et al.* Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* **121**, 335-348 (2005).
198. Anderberg, C., *et al.* Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts. *Cancer research* **69**, 369-378 (2009).
199. Karnoub, A.E., *et al.* Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* **449**, 557-563 (2007).
200. Bhowmick, N.A., *et al.* TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. *Science* **303**, 848-851 (2004).
201. Camps, J.L., *et al.* Fibroblast-mediated acceleration of human epithelial tumor growth in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 75-79 (1990).
202. Gleave, M., Hsieh, J.T., Gao, C.A., von Eschenbach, A.C. & Chung, L.W. Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. *Cancer research* **51**, 3753-3761 (1991).
203. Erez, N., Truitt, M., Olson, P., Arron, S.T. & Hanahan, D. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. *Cancer cell* **17**, 135-147 (2010).
204. Pietras, K., Pahler, J., Bergers, G. & Hanahan, D. Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting. *PLoS medicine* **5**, e19 (2008).

205. Augsten, M., *et al.* CXCL14 is an autocrine growth factor for fibroblasts and acts as a multi-modal stimulator of prostate tumor growth. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 3414-3419 (2009).
206. Calon, A., *et al.* Dependency of colorectal cancer on a TGF-beta-driven program in stromal cells for metastasis initiation. *Cancer cell* **22**, 571-584 (2012).
207. Pena, C., *et al.* STC1 expression by cancer-associated fibroblasts drives metastasis of colorectal cancer. *Cancer research* **73**, 1287-1297 (2013).
208. Kim, J.W., *et al.* Loss of fibroblast HIF-1alpha accelerates tumorigenesis. *Cancer research* **72**, 3187-3195 (2012).
209. Madsen, C.D., *et al.* Hypoxia and loss of PHD2 inactivate stromal fibroblasts to decrease tumour stiffness and metastasis. *EMBO reports* **16**, 1394-1408 (2015).
210. Kuchnio, A., *et al.* The Cancer Cell Oxygen Sensor PHD2 Promotes Metastasis via Activation of Cancer-Associated Fibroblasts. *Cell reports* **12**, 992-1005 (2015).
211. Breit, S.N., *et al.* The TGF-beta superfamily cytokine, MIC-1/GDF15: a pleotrophic cytokine with roles in inflammation, cancer and metabolism. *Growth Factors* **29**, 187-195 (2011).
212. Zhang, X.H., *et al.* Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. *Cell* **154**, 1060-1073 (2013).
213. Sanz-Moreno, V., *et al.* ROCK and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma. *Cancer cell* **20**, 229-245 (2011).
214. Paulsson, J. & Micke, P. Prognostic relevance of cancer-associated fibroblasts in human cancer. *Seminars in cancer biology* **25**, 61-68 (2014).
215. Auvinen, P., *et al.* Increased hyaluronan content and stromal cell CD44 associate with HER2 positivity and poor prognosis in human breast cancer. *International journal of cancer. Journal international du cancer* **132**, 531-539 (2013).
216. Ioachim, E., *et al.* Immunohistochemical expression of extracellular matrix components tenascin, fibronectin, collagen type IV and laminin in breast cancer: their prognostic value and role in tumour invasion and progression. *Eur J Cancer* **38**, 2362-2370 (2002).
217. Ariga, N., Sato, E., Ohuchi, N., Nagura, H. & Ohtani, H. Stromal expression of fibroblast activation protein/seprase, a cell membrane serine proteinase and gelatinase, is associated with longer survival in patients with invasive ductal carcinoma of breast. *International journal of cancer. Journal international du cancer* **95**, 67-72 (2001).
218. Qian, N., *et al.* Prognostic significance of tumor/stromal caveolin-1 expression in breast cancer patients. *Cancer science* **102**, 1590-1596 (2011).
219. Goetz, J.G., *et al.* Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. *Cell* **146**, 148-163 (2011).
220. Simpkins, S.A., Hanby, A.M., Holliday, D.L. & Speirs, V. Clinical and functional significance of loss of caveolin-1 expression in breast cancer-associated fibroblasts. *The Journal of pathology* **227**, 490-498 (2012).
221. Schoppmann, S.F., *et al.* Podoplanin-expressing cancer-associated fibroblasts are associated with poor prognosis in invasive breast cancer. *Breast cancer research and treatment* **134**, 237-244 (2012).
222. Folgueira, M.A., *et al.* Markers of breast cancer stromal fibroblasts in the primary tumour site associated with lymph node metastasis: a systematic review including our case series. *Bioscience reports* **33**(2013).
223. Calon, A., *et al.* Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nature genetics* **47**, 320-329 (2015).
224. Finak, G., *et al.* Stromal gene expression predicts clinical outcome in breast cancer. *Nature medicine* **14**, 518-527 (2008).

225. Frings, O.*, et al.* Prognostic significance in breast cancer of a gene signature capturing stromal PDGF signaling. *The American journal of pathology* **182**, 2037-2047 (2013).
226. Balkwill, F. Cancer and the chemokine network. *Nature reviews. Cancer* **4**, 540-550 (2004).
227. Zlotnik, A. & Yoshie, O. Chemokines: a new classification system and their role in immunity. *Immunity* **12**, 121-127 (2000).
228. Zlotnik, A. & Yoshie, O. The chemokine superfamily revisited. *Immunity* **36**, 705-716 (2012).
229. Murphy, P.M.*, et al.* International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacological reviews* **52**, 145-176 (2000).
230. Bonecchi, R.*, et al.* Non-signaling chemokine receptors: mechanism of action and role in vivo. *Journal of neuroimmunology* **198**, 14-19 (2008).
231. Bachelerie, F.*, et al.* New nomenclature for atypical chemokine receptors. *Nature immunology* **15**, 207-208 (2014).
232. Mantovani, A., Bonecchi, R. & Locati, M. Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nature reviews. Immunology* **6**, 907-918 (2006).
233. Bonecchi, R., Savino, B., Borroni, E.M., Mantovani, A. & Locati, M. Chemokine decoy receptors: structure-function and biological properties. *Current topics in microbiology and immunology* **341**, 15-36 (2010).
234. Rossi, D. & Zlotnik, A. The biology of chemokines and their receptors. *Annual review of immunology* **18**, 217-242 (2000).
235. Viola, A. & Luster, A.D. Chemokines and their receptors: drug targets in immunity and inflammation. *Annual review of pharmacology and toxicology* **48**, 171-197 (2008).
236. Lagerstrom, M.C. & Schioth, H.B. Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nature reviews. Drug discovery* **7**, 339-357 (2008).
237. Murdoch, C. & Finn, A. Chemokine receptors and their role in inflammation and infectious diseases. *Blood* **95**, 3032-3043 (2000).
238. Locht, C., Coutte, L. & Mielcarek, N. The ins and outs of pertussis toxin. *The FEBS journal* **278**, 4668-4682 (2011).
239. Johrer, K.*, et al.* Tumour-immune cell interactions modulated by chemokines. *Expert opinion on biological therapy* **8**, 269-290 (2008).
240. Thelen, M. Dancing to the tune of chemokines. *Nature immunology* **2**, 129-134 (2001).
241. Teicher, B.A. & Fricker, S.P. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 2927-2931 (2010).
242. Nomiyama, H. & Yoshie, O. Functional roles of evolutionary conserved motifs and residues in vertebrate chemokine receptors. *Journal of leukocyte biology* **97**, 39-47 (2015).
243. Graham, G.J., Locati, M., Mantovani, A., Rot, A. & Thelen, M. The biochemistry and biology of the atypical chemokine receptors. *Immunology letters* **145**, 30-38 (2012).
244. Hoffmann, F.*, et al.* Rapid uptake and degradation of CXCL12 depend on CXCR7 carboxyl-terminal serine/threonine residues. *The Journal of biological chemistry* **287**, 28362-28377 (2012).
245. Levoye, A., Balabanian, K., Baleux, F., Bachelerie, F. & Lagane, B. CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood* **113**, 6085-6093 (2009).

246. Odemis, V., *et al.* The presumed atypical chemokine receptor CXCR7 signals through G(i/o) proteins in primary rodent astrocytes and human glioma cells. *Glia* **60**, 372-381 (2012).
247. Nibbs, R.J., *et al.* The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *The American journal of pathology* **158**, 867-877 (2001).
248. Martinez de la Torre, Y., *et al.* Protection against inflammation- and autoantibody-caused fetal loss by the chemokine decoy receptor D6. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 2319-2324 (2007).
249. McKimmie, C.S. & Graham, G.J. Leucocyte expression of the chemokine scavenger D6. *Biochemical Society transactions* **34**, 1002-1004 (2006).
250. Neil, S.J., *et al.* The promiscuous CC chemokine receptor D6 is a functional coreceptor for primary isolates of human immunodeficiency virus type 1 (HIV-1) and HIV-2 on astrocytes. *Journal of virology* **79**, 9618-9624 (2005).
251. Nibbs, R.J., Wylie, S.M., Pragnell, I.B. & Graham, G.J. Cloning and characterization of a novel murine beta chemokine receptor, D6. Comparison to three other related macrophage inflammatory protein-1alpha receptors, CCR-1, CCR-3, and CCR-5. *The Journal of biological chemistry* **272**, 12495-12504 (1997).
252. Fra, A.M., *et al.* Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. *J Immunol* **170**, 2279-2282 (2003).
253. Borroni, E.M., *et al.* beta-arrestin-dependent activation of the cofilin pathway is required for the scavenging activity of the atypical chemokine receptor D6. *Science signaling* **6**, ra30 31-11, S31-33 (2013).
254. Singh, S., Sadanandam, A. & Singh, R.K. Chemokines in tumor angiogenesis and metastasis. *Cancer metastasis reviews* **26**, 453-467 (2007).
255. Muller, A., *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**, 50-56 (2001).
256. O'Hayre, M., Salanga, C.L., Handel, T.M. & Allen, S.J. Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. *The Biochemical journal* **409**, 635-649 (2008).
257. Bailey, C., *et al.* Chemokine expression is associated with the accumulation of tumour associated macrophages (TAMs) and progression in human colorectal cancer. *Clinical & experimental metastasis* **24**, 121-130 (2007).
258. Saji, H., *et al.* Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma. *Cancer* **92**, 1085-1091 (2001).
259. Ueno, T., *et al.* Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **6**, 3282-3289 (2000).
260. Luboshits, G., *et al.* Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. *Cancer research* **59**, 4681-4687 (1999).
261. Gazzaniga, S., *et al.* Targeting tumor-associated macrophages and inhibition of MCP-1 reduce angiogenesis and tumor growth in a human melanoma xenograft. *The Journal of investigative dermatology* **127**, 2031-2041 (2007).
262. Monti, P., *et al.* The CC chemokine MCP-1/CCL2 in pancreatic cancer progression: regulation of expression and potential mechanisms of antimalignant activity. *Cancer research* **63**, 7451-7461 (2003).
263. Strieter, R.M., *et al.* Cancer CXC chemokine networks and tumour angiogenesis. *Eur J Cancer* **42**, 768-778 (2006).

264. Balabanian, K.*, et al.* The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *The Journal of biological chemistry* **280**, 35760-35766 (2005).
265. Burns, J.M.*, et al.* A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *The Journal of experimental medicine* **203**, 2201-2213 (2006).
266. Burger, J.A. & Kipps, T.J. CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. *Blood* **107**, 1761-1767 (2006).
267. Addison, C.L.*, et al.* The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol* **165**, 5269-5277 (2000).
268. Singh, S., Singh, A.P., Sharma, B., Owen, L.B. & Singh, R.K. CXCL8 and its cognate receptors in melanoma progression and metastasis. *Future Oncol* **6**, 111-116 (2010).
269. Matsuo, Y.*, et al.* CXCL8/IL-8 and CXCL12/SDF-1alpha co-operatively promote invasiveness and angiogenesis in pancreatic cancer. *International journal of cancer. Journal international du cancer* **124**, 853-861 (2009).
270. Acharyya, S.*, et al.* A CXCL1 paracrine network links cancer chemoresistance and metastasis. *Cell* **150**, 165-178 (2012).
271. Lazennec, G. & Richmond, A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends in molecular medicine* **16**, 133-144 (2010).
272. Zlotnik, A., Burkhardt, A.M. & Homey, B. Homeostatic chemokine receptors and organ-specific metastasis. *Nature reviews. Immunology* **11**, 597-606 (2011).
273. Sobolik, T.*, et al.* CXCR4 drives the metastatic phenotype in breast cancer through induction of CXCR2 and activation of MEK and PI3K pathways. *Molecular biology of the cell* **25**, 566-582 (2014).
274. Visciano, C.*, et al.* Mast cells induce epithelial-to-mesenchymal transition and stem cell features in human thyroid cancer cells through an IL-8-Akt-Slug pathway. *Oncogene* **34**, 5175-5186 (2015).
275. Wu, Y.C., Tang, S.J., Sun, G.H. & Sun, K.H. CXCR7 mediates TGFbeta1-promoted EMT and tumor-initiating features in lung cancer. *Oncogene* **35**, 2123-2132 (2016).
276. Harata-Lee, Y.*, et al.* The atypical chemokine receptor CCX-CKR regulates metastasis of mammary carcinoma via an effect on EMT. *Immunology and cell biology* **92**, 815-824 (2014).
277. Allinen, M.*, et al.* Molecular characterization of the tumor microenvironment in breast cancer. *Cancer cell* **6**, 17-32 (2004).
278. Murakami, T.*, et al.* Immune evasion by murine melanoma mediated through CC chemokine receptor-10. *The Journal of experimental medicine* **198**, 1337-1347 (2003).
279. Wiley, H.E., Gonzalez, E.B., Maki, W., Wu, M.T. & Hwang, S.T. Expression of CC chemokine receptor-7 and regional lymph node metastasis of B16 murine melanoma. *Journal of the National Cancer Institute* **93**, 1638-1643 (2001).
280. Murakami, T.*, et al.* Expression of CXC chemokine receptor-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. *Cancer research* **62**, 7328-7334 (2002).
281. Zhang, X.H.*, et al.* Latent bone metastasis in breast cancer tied to Src-dependent survival signals. *Cancer cell* **16**, 67-78 (2009).
282. Li, A.*, et al.* Overexpression of CXCL5 is associated with poor survival in patients with pancreatic cancer. *The American journal of pathology* **178**, 1340-1349 (2011).
283. Gao, Q.*, et al.* CXCR6 upregulation contributes to a proinflammatory tumor microenvironment that drives metastasis and poor patient outcomes in hepatocellular carcinoma. *Cancer research* **72**, 3546-3556 (2012).

284. Izumi, D.*, et al.* CXCL12/CXCR4 activation by cancer-associated fibroblasts promotes integrin beta1 clustering and invasiveness in gastric cancer. *International journal of cancer. Journal international du cancer* **138**, 1207-1219 (2016).
285. Scholten, D.J.*, et al.* Pharmacological modulation of chemokine receptor function. *British journal of pharmacology* **165**, 1617-1643 (2012).
286. Ao, M.*, et al.* Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. *Cancer research* **67**, 4244-4253 (2007).
287. Wang, J.*, et al.* Characterization of phosphoglycerate kinase-1 expression of stromal cells derived from tumor microenvironment in prostate cancer progression. *Cancer research* **70**, 471-480 (2010).
288. Daly, A.J., McIlreavey, L. & Irwin, C.R. Regulation of HGF and SDF-1 expression by oral fibroblasts--implications for invasion of oral cancer. *Oral oncology* **44**, 646-651 (2008).
289. Moskovits, N., Kalinkovich, A., Bar, J., Lapidot, T. & Oren, M. p53 Attenuates cancer cell migration and invasion through repression of SDF-1/CXCL12 expression in stromal fibroblasts. *Cancer research* **66**, 10671-10676 (2006).
290. Kojima, K.*, et al.* p53 activation of mesenchymal stromal cells partially abrogates microenvironment-mediated resistance to FLT3 inhibition in AML through HIF-1alpha-mediated down-regulation of CXCL12. *Blood* **118**, 4431-4439 (2011).
291. Tsuyada, A.*, et al.* CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. *Cancer research* **72**, 2768-2779 (2012).
292. Jung, Y.*, et al.* Recruitment of mesenchymal stem cells into prostate tumours promotes metastasis. *Nature communications* **4**, 1795 (2013).
293. Smith, M.C.*, et al.* CXCR4 regulates growth of both primary and metastatic breast cancer. *Cancer research* **64**, 8604-8612 (2004).
294. Huising, M.O., van der Meulen, T., Flik, G. & Verburg-van Kemenade, B.M. Three novel carp CXC chemokines are expressed early in ontogeny and at nonimmune sites. *European journal of biochemistry / FEBS* **271**, 4094-4106 (2004).
295. DeVries, M.E.*, et al.* Defining the origins and evolution of the chemokine/chemokine receptor system. *J Immunol* **176**, 401-415 (2006).
296. Hromas, R.*, et al.* Cloning of BRAK, a novel divergent CXC chemokine preferentially expressed in normal versus malignant cells. *Biochemical and biophysical research communications* **255**, 703-706 (1999).
297. Frederick, M.J.*, et al.* In vivo expression of the novel CXC chemokine BRAK in normal and cancerous human tissue. *The American journal of pathology* **156**, 1937-1950 (2000).
298. Sleeman, M.A.*, et al.* B cell- and monocyte-activating chemokine (BMAC), a novel non-ELR alpha-chemokine. *International immunology* **12**, 677-689 (2000).
299. Hara, T. & Tanegashima, K. Pleiotropic functions of the CXC-type chemokine CXCL14 in mammals. *Journal of biochemistry* **151**, 469-476 (2012).
300. Kurth, I.*, et al.* Monocyte selectivity and tissue localization suggests a role for breast and kidney-expressed chemokine (BRAK) in macrophage development. *The Journal of experimental medicine* **194**, 855-861 (2001).
301. Shellenberger, T.D.*, et al.* BRAK/CXCL14 is a potent inhibitor of angiogenesis and a chemotactic factor for immature dendritic cells. *Cancer research* **64**, 8262-8270 (2004).
302. Shurin, G.V.*, et al.* Loss of new chemokine CXCL14 in tumor tissue is associated with low infiltration by dendritic cells (DC), while restoration of human CXCL14 expression in tumor cells causes attraction of DC both in vitro and in vivo. *J Immunol* **174**, 5490-5498 (2005).

303. Starnes, T., *et al.* The chemokine CXCL14 (BRAK) stimulates activated NK cell migration: implications for the downregulation of CXCL14 in malignancy. *Experimental hematology* **34**, 1101-1105 (2006).
304. Lu, J., Chatterjee, M., Schmid, H., Beck, S. & Gawaz, M. CXCL14 as an emerging immune and inflammatory modulator. *J Inflamm (Lond)* **13**, 1 (2016).
305. Tessema, M., *et al.* Re-expression of CXCL14, a common target for epigenetic silencing in lung cancer, induces tumor necrosis. *Oncogene* **29**, 5159-5170 (2010).
306. Ozawa, S., *et al.* Restoration of BRAK / CXCL14 gene expression by gefitinib is associated with antitumor efficacy of the drug in head and neck squamous cell carcinoma. *Cancer science* **100**, 2202-2209 (2009).
307. Schwarze, S.R., Luo, J., Isaacs, W.B. & Jarrard, D.F. Modulation of CXCL14 (BRAK) expression in prostate cancer. *The Prostate* **64**, 67-74 (2005).
308. Wente, M.N., *et al.* CXCL14 expression and potential function in pancreatic cancer. *Cancer letters* **259**, 209-217 (2008).
309. Song, E.Y., Shurin, M.R., Tourkova, I.L., Gutkin, D.W. & Shurin, G.V. Epigenetic mechanisms of promigratory chemokine CXCL14 regulation in human prostate cancer cells. *Cancer research* **70**, 4394-4401 (2010).
310. Cao, B., *et al.* Epigenetic silencing of CXCL14 induced colorectal cancer migration and invasion. *Discovery medicine* **16**, 137-147 (2013).
311. Gu, X.L., *et al.* Expression of CXCL14 and its anticancer role in breast cancer. *Breast cancer research and treatment* **135**, 725-735 (2012).
312. Ozawa, S., Kato, Y., Kubota, E. & Hata, R. BRAK/CXCL14 expression in oral carcinoma cells completely suppresses tumor cell xenografts in SCID mouse. *Biomed Res* **30**, 315-318 (2009).
313. Hata, R., *et al.* Suppressed rate of carcinogenesis and decreases in tumour volume and lung metastasis in CXCL14/BRAK transgenic mice. *Scientific reports* **5**, 9083 (2015).
314. Strieter, R.M., *et al.* The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *The Journal of biological chemistry* **270**, 27348-27357 (1995).
315. Kijowski, J., *et al.* The SDF-1-CXCR4 axis stimulates VEGF secretion and activates integrins but does not affect proliferation and survival in lymphohematopoietic cells. *Stem Cells* **19**, 453-466 (2001).
316. Izukuri, K., *et al.* Chemokine CXCL14/BRAK transgenic mice suppress growth of carcinoma cell transplants. [corrected]. *Transgenic research* **19**, 1109-1117 (2010).
317. Pelicano, H., *et al.* Mitochondrial dysfunction and reactive oxygen species imbalance promote breast cancer cell motility through a CXCL14-mediated mechanism. *Cancer research* **69**, 2375-2383 (2009).
318. Zeng, J., *et al.* Chemokine CXCL14 is associated with prognosis in patients with colorectal carcinoma after curative resection. *Journal of translational medicine* **11**, 6 (2013).
319. Lu, J., *et al.* IRX1 hypomethylation promotes osteosarcoma metastasis via induction of CXCL14/NF-kappaB signaling. *The Journal of clinical investigation* **125**, 1839-1856 (2015).
320. Takiguchi, S., *et al.* Involvement of CXCL14 in osteolytic bone metastasis from lung cancer. *International journal of oncology* **44**, 1316-1324 (2014).
321. Williams, K.A., *et al.* A systems genetics approach identifies CXCL14, ITGAX, and LPCAT2 as novel aggressive prostate cancer susceptibility genes. *PLoS genetics* **10**, e1004809 (2014).
322. Riester, M., *et al.* Risk prediction for late-stage ovarian cancer by meta-analysis of 1525 patient samples. *Journal of the National Cancer Institute* **106**(2014).

323. Chiu, S.H., Chen, C.C. & Lin, T.H. Using support vector regression to model the correlation between the clinical metastases time and gene expression profile for breast cancer. *Artificial intelligence in medicine* **44**, 221-231 (2008).
324. Oler, G.*, et al.* Gene expression profiling of papillary thyroid carcinoma identifies transcripts correlated with BRAF mutational status and lymph node metastasis. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14**, 4735-4742 (2008).
325. Lin, K.*, et al.* Expression and effect of CXCL14 in colorectal carcinoma. *Mol Med Rep* **10**, 1561-1568 (2014).
326. Marsh, T., Pietras, K. & McAllister, S.S. Fibroblasts as architects of cancer pathogenesis. *Biochimica et biophysica acta* **1832**, 1070-1078 (2013).
327. Bissell, M.J. & Hines, W.C. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nature medicine* **17**, 320-329 (2011).
328. De Minicis, S.*, et al.* Gene expression profiles during hepatic stellate cell activation in culture and in vivo. *Gastroenterology* **132**, 1937-1946 (2007).
329. Kurth, I.*, et al.* Monocyte selectivity and tissue localization suggests a role for breast and kidney-expressed chemokine (BRAK) in macrophage development. *J Exp Med* **194**, 855-861 (2001).
330. Tanegashima, K.*, et al.* CXCL14 is a natural inhibitor of the CXCL12-CXCR4 signaling axis. *FEBS letters* **587**, 1731-1735 (2013).
331. Otte, M.*, et al.* CXCL14 is no direct modulator of CXCR4. *FEBS letters* **588**, 4769-4775 (2014).