‘Predicting breast cancer metastasis; formation of the pre-metastatic niche’

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Abstract

The leading cause of death in breast cancer patients is tumor metastasis. This process includes several steps that all need to be completed successfully to develop distant metastatic disease. Tumor cells disseminate from the primary tumor and cross multiple barriers like the basement membrane and extracellular matrix. Subsequently, tumor cells enter the circulation by a process called intravasation, and they are transported throughout the body. At specific sites, tumor cells adhere to the blood vessel walls, breach the vascular endothelium (extravasation) and colonize target organs. Breast cancer shows striking tissue tropism for the formation of metastases in lung, bone, brain and liver. The molecular mechanisms underlying this specificity are not yet known, but most likely it is caused by an interplay between tumor cells and the microenvironment at target organs. Studies have shown that, before arrival of tumor cells, the microenvironment in target organs is modulated to form a permissive 'pre-metastatic niche'. Unraveling the mechanisms underlying the formation of this 'pre-metastatic niche' might contribute to our ability to predict breast cancer metastasis and the development of specific anti-cancer drugs and therapies.

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Introduction

After decades of research and millions of dollars, breast cancer still remains the leading cause of cancer mortality in women (28% of all cancer deaths in women is due to breast cancer). It is estimated that in the past year in the United States 207,090 women were diagnosed with breast cancer and 39,840 women died from this disease[1]. The majority of these breast cancer deaths is due to malignant disease whereby detached tumor cells from the primary tumor spread throughout the body and form distant metastases[2]. However, most treatment strategies used nowadays, including surgery and radiation therapy, are developed to target the primary tumor. We need to appreciate the fact that cancer is a systemic disease, and treatment of the primary tumor is not sufficient to cure most cancer patients. Therefore, the focus in cancer research recently showed a slight shift towards unraveling the complex molecular pathways underlying tumor metastasis. Advanced microarray studies revealing gene-expression signatures of primary breast tumors are now able to predict the likelihood of the formation of distant metastases[3]. Like most cancer types, breast cancer displays specificity for developing metastases in distant organs like lymph, bone, lung and liver. A peculiar fact is that disseminated breast cancer cells often remain dormant for years and are able to cause recurrence even 10 years or more after detection[4]. The factors underlying this dormancy have not been elucidated yet, although it has been suggested that tumor cells remain in a specific 'niche' allowing them to survive at a distant site. As early as in 1889 the English surgeon Stephen Paget proposed his 'seed and soil' hypothesis which states that metastasis depends on cross-talk between selected cancer cells (the 'seeds') and a specific organ microenvironment (the 'soil')[5]. More recently, evidence for the existence of a so called 'pre-metastatic niche' was published by R.N. Kaplan and D. Lyden[6], and since then a growing amount of promising data has been published on this topic. In this review we aim to give an overview of our growing ability to predict breast cancer metastasis by studying the formation of a pre-metastatic niche.

Metastasis: a step-by-step process

The process of metastasis consists of several steps that all need to be completed successfully to form a metastatic tumor (Figure 1). Tumor cells need to detach from the primary tumor and intravasate into blood vessels to enter the circulation. Once in the circulation, cells can be transported over long distances towards other organs. At particular sites dependent on the type of cancer, cells adhere to blood vessel walls, extravasate and seed in target organs. Some cells remain dormant for a long time before they start the formation of metastatic foci. It is likely that the interaction with the microenvironment contributes to the behavior of tumor cells in these distant organs. This will be discussed in more detail later on in this review.

Tumor heterogeneity

The first step in tumor progression is most likely favored by the heterogeneity of cells forming a primary tumor (Figure 1a). By obtaining mutations in their DNA tumor cells show uncontrolled growth. The intrinsic genomic instability of tumor cells causes an increase in mutation frequency, and therefore chromosomal gains and losses as well as chromosomal recombination events are often observed in cancer cells. For example, a loss-of-function mutation in the tumor suppressor protein p53 is observed in at least 50% of the cancers[7, 8]. Mutations in this DNA damage response gene result in escape of cell growth arrest and apoptosis, leading to an accumulation of cells with DNA damage. Accordingly, impaired cellular processes like improper cell cycle progression, inactivation of DNA repair mechanisms and epigenetic regulation of genes, contribute to malignant transformation. Not only genomic aberrations, but also the tumor morphology itself, causes heterogeneity of tumor cells[9]. During tumor growth, a population of cells becomes hypoxic caused by an increased distance from blood vessels providing oxygen and nutrients. Cellular hypoxia causes activation of the transcription factor Hypoxia Inducible Factor (HIF) that induces an adaptation mechanism[10] for cells to survive low oxygen levels[11, 12]. The subsequent shift from aerobe to anaerobe metabolism allows hypoxic cells to survive the stringent conditions and to acquire metastatic potential. Namely, HIF-dependent upregulation of lysyl oxidase (LOX) has been shown in to correlate with poor overall survival of breast cancer patients by promoting an invasive tumor cell phenotype[13-15]. Another process which is triggered by hypoxia is the formation of new blood vessels, or angiogenesis (Figure 1b). By HIF-dependent transcriptional activation of the gene encoding vascular endothelial growth factor (VEGF) new blood vessels are formed in the expanding tumor mass to restore the levels of oxygen[16-18]. This intratumoral vasculature is often leaky and shows malformations, and might be used by disseminated tumor cells to enter the circulation[19].

Detachment and invasion

In the subsequent step of the metastatic process, tumor cells lose their architecture and polarity and obtain a migratory capacity to disseminate from the primary tumor and spread throughout the body (Figure 1c). This migratory behavior is regulated by an interplay of adhesive and proteolytic mechanisms, as well as by changes in expression of cytoskeletal proteins. Individual tumor cells may acquire the ability to detach from their neighboring cells by downregulation of cell interaction molecules like cadherins and integrins. The process in which epithelial cells transform to a motile mesenchymal phenotype is also known as epithelial-to-mesenchymal transition (EMT). Several *in vitro* and clinical studies have shown that a loss of expression of the cell-cell adhesion protein E-cadherin in breast carcinoma cells correlates with an invasive phenotype[20-23]. More specifically, it seems that in epithelial cells undergoing EMT, promoter methylation of the gene encoding E-cadherin, instead of mutation, gives rise to a migratory fibroblast-like phenotype[24, 25]. During migration, tumor cells have to cross connective tissue barriers like the extracellular matrix (ECM) and the basement membrane (BM) as well as the interstitial stroma. The ECM consists of collagens, laminins and fibronectin and serves as a framework for cells along which they can attach and migrate. Migration of tumor cells through the ECM can occur in different patterns (reviewed in [26, 27]). A 'fibroblast-like' form of migration is characterized by cellular actin-rich protrusions containing integrin receptor complexes and matrix metalloproteinases (MMPs), that facilitate crossing the ECM by proteolytic matrix degradation[26]. Single cell migration can be described as 'amoeboid' in which integrins and cell adhesion molecules (CAMs)

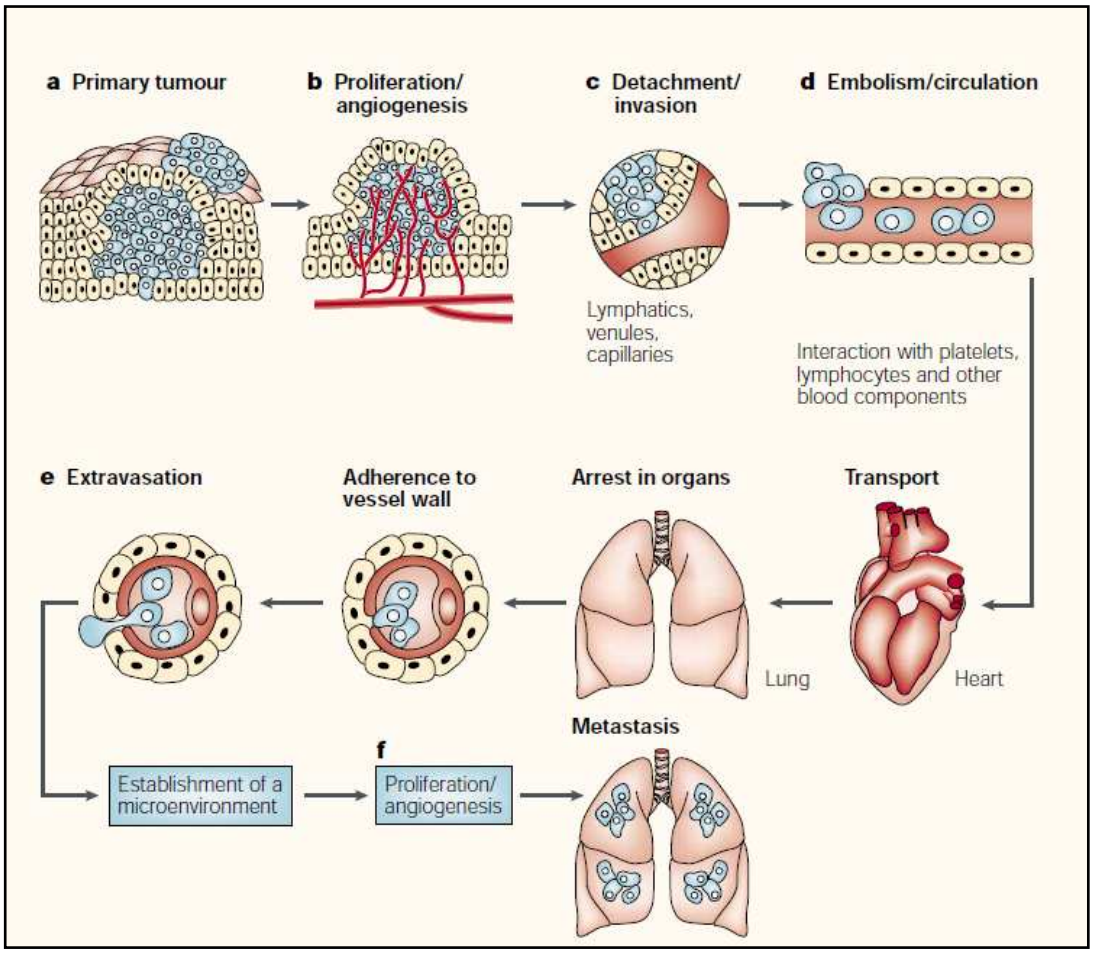

Figure 1. Tumor metastasis is a step-by-step process. (a) Tumor growth is initiated by transformed cells. Nutrients and oxygen are provided by diffusion. (b) Proliferation causes tumor growth and a fraction of cells become hypoxic. HIF-dependent angiogenesis is required for tumor growth beyond 1-2 mm^3. (c) Loss of cell junctions leads to detachment of cells and intravasation into the blood stream or lymphatics (d). Circulating cancer cells are transported towards specific organs. Here they get trapped in capillary beds, adhere to blood vessel walls and extravasate (e). A supportive microenvironment is required for successful seeding, colonization and the formation of metastases (f). Figure adapted from [38].

are evenly distributed over the cell surface. Because of the lack of matrix remodeling molecules to pave the way through the ECM, the cell has to change its shape to crawl through the ECM barrier. Understanding the complexities of the interplay between epithelium and the surrounding stromal compartment is of great importance for cancer biology. Although the precise mechanism is still unknown, it is accepted that the stroma is an important contributor to tumor progression[28, 29]. Aberrations in the ECM integrity and composition are often associated with breast carcinogenesis[30, 31]. For example, breast cancer often shows dense fibrosis around the tumor, also known as desmoplasia. Fibrotic collagen type I disposition causes activation of MMP-2 expression in tumor cells resulting in invasive behavior at the primary or metastatic site[32]. Also MMP-9 expression is implicated in cancer cell invasion and progression to metastasis by priming the microenvironment in the lung, thereby facilitating infiltration of tumor cells[33]. Moreover, gene expression studies revealed that upregulation of the fibrillar collagen gene correlates with metastatic phenotype in breast cancer[34].

Intravasation

After dissemination from the primary tumor, cancer cells enter the circulation by a process called intravasation (Figure 1c,d). As described earlier, the perturbed intratumoral vasculature provides an escape route for tumor cells to disseminate to distant organs. An elegant study using intravital imaging showed the intravasation and metastatic potential of breast adenocarcinoma cells in rats[35]. GFP-tagged metastatic and non-metastatic breast adenocarcinoma cell lines were injected orthotopically in the inguinal mammary fat pad and allowed to form tumors over 2.5 weeks. Blood was withdrawn from these animals and using a colony forming assay they showed that the number of viable tumor cells in the blood was higher in animals carrying metastatic tumors compared to animals carrying non-metastatic tumors. Data analysis based on GFP expression revealed a correlation between the tumor cell density in the blood and the number of GFP^+ metastatic foci in the lungs[35]. These results suggest that metastatic tumors are able to enter the circulation more efficiently than non-metastatic tumors irrespective of tumor size. Tumor cells can also enter the blood circulation indirectly via the lymphatic system. Skin carcinomas overexpressing VEGF-C induce expansion of the lymphatic network within the sentinel lymph nodes which promotes metastasis to distant organs[36]. Despite many studies, it is not clear whether blood vessels or lymphatics are the main route responsible for tumor metastasis.

Extravasation and homing

The subsequent step of leaving the circulation, and entry and outgrowth in target organs is also called extravasation and homing (Figure 1e). Studies dating back 40 years used radioactive labeling of cells and showed that 24 hrs after injection of tumor cells in the circulation of mice, only 0.1% of tumor cells is still viable and 0.01% is able to form metastatic foci in distant organs[37, 38]. More recent studies with melanoma cells reveal that a minor fraction of tumor cells is destroyed in the circulation probably by the immune system. Although the remaining large fraction of cells extravasates at distant sites, only several manage to form metastatic lesions[39, 40]. Thus, metastasis seems to be an inefficient process. Moreover, results from clinical studies in breast cancer patients suggest that metastatic foci after surgery of the primary tumor do not show continuous growth over time, but rather show a form of tumor dormancy[41]. Recurrence of tumors can occur years after treatment[42]. Especially breast cancer is known to have a long period of dormancy. Although most recurrences occur within 10 years after mastectomy, in some cases the tumor reoccurs after 26-45 years[42]. The mechanisms underlying tumor cell dormancy are still not very well understood. It has been proposed that tumor cells remain in a specific regulatory niche that maintains them in a quiescent stem cell-like state[43]. Indeed, dormant tumor cells have been found to remain in G_0/G_1 growth arrest as shown by negative staining for proliferation markers including Ki67[44, 45]. Obtaining a stem cell-like state with a low proliferation capacity might make tumor cells invisible for the immune system and anti-cancer drugs which target highly proliferating cells. Also, the failure of metastatic foci to produce angiogenic factors will prevent further growth[46]. Experimental studies in mice have shown that primary tumors produce angiogenesis inhibitory factors that maintain distant micrometastases in a dormant and avascular state[47]. After removal of the primary tumor an angiogenic switch occurs which increases the proliferative capacity of micrometastatic foci allowing them to grow and form macrometastases (Figure 1f). However, no evidence for such factors was found in humans.

The likelihood that metastatic foci at distant organs remain dormant for many years makes it more difficult to predict at what stage in tumor progression metastasis occurs. Over the years scientists default to the assumption that metastasis occurs as a late event in tumor progression, however, this topic is still under investigation. Several studies show that metastasis can occur in pre-invasive lesions; disseminated tumor cells have been detected in bone marrow of breast cancer patients early

in tumor progression[48, 49]. Single-cell comparative genomic hybridization analysis revealed that these cells have acquired additional genomic aberrations when compared to the genome of cells in the primary tumor[49, 50]. The size of the primary tumor does not seem to correlate with metastatic spread, since dissemination of tumor cells was also observed in small tumors[50]. Genomic studies on the expression profile of primary tumor cells seem to be able to predict the likelihood of spread throughout the body[34, 51, 52]. But how specific can we predict tumor metastasis? Breast cancer shows striking specificity for the development of metastatic foci in lung and bone marrow, and to a lower extend in liver and brain[38]. Is this tropism caused by the fact that these organs are highly vascularized and disseminated tumor cells get trapped in the capillary beds? Or is there another mechanism at work that determines the distant sites of metastasis?

To answer these questions many researchers reached back to Paget's 'seed and soil' hypothesis suggesting that cancer cells need a supportive microenvironment to develop into metastases. By unraveling the secrets of the 'pre-metastatic niche', better treatment strategies to prevent cancer metastases might be developed.

Formation of the pre-metastatic niche

Researchers have been studying cancer metastases for more than 100 years. In 1889, the English surgeon Stephen Paget published his 'seed and soil' hypothesis to explain the distribution of metastases over the body. After studying over 900 autopsy reports from patients with different types of primary tumors he concluded that metastasis occurs in a non-random pattern, which was in contrast with what was assumed at that time. Paget proposed that tumor cells (the 'seeds') have affinity for a microenvironment in specific distant organs (the 'soil'), and interaction between the 'seed and soil' determines the particular sites of metastases[5]. Most studies have been designed to unravel the genetic changes underlying the transformation of particular primary tumor cells or the 'seeds'. The prevailing model is that subsets of tumor cells acquire metastatic potential late in tumor progression which allows dissemination and formation of distant metastases. However, the interaction with stromal cells at the distant site turns out to be at least equally important in this process. Therefore, the formation of metastases is tightly regulated by both intrinsic factors in the tumor cells (the 'seeds') and extrinsic factors from the microenvironment (the 'soil').

Dynamic interplay of intrinsic and extrinsic factors

Primary tumor cells express a specific array of genes that might predict metastasis. Several groups have published gene expression signatures to predict the site of metastasis and even prognosis of patients[34, 52-54]. However, the contributing role of the microenvironment in tumor metastasis should not be underestimated. Tumor cells instruct stromal cells at target organs to form a permissive microenvironment that supports the survival and growth of disseminated tumor cells either by direct cell-cell contact or secreting factors[55]. Stromal cells, including fibroblasts, infiltrating immune cells and bone marrow-derived stem and progenitor cells, respond by the production and secretion of a variety of growth factors, chemokines and cytokines. In the following sections we will highlight some of them.

Tissue stroma contains many cell types, but the majority is constituted by fibroblasts. Several studies using organotypic culture have shown that carcinoma-associated fibroblasts (CAFs) present in the tumor stroma, have tumor-promoting properties as compared to normal fibroblasts[56-58]. CAFs can increase the migratory ability of a breast cancer cell line by inducing EMT[56]. Also, CAFs might contribute to localized deposition of fibronectin and thereby priming tissue for circulating bone marrow-derived cells (BMDC) and tumor cells[6]. Moreover, CAFs were found to express a truncated isoform of fibronectin which results in a loss-of-function of the protein and detachment of tumor cells from their ECM scaffold[57].

Studies revealed that one of the earliest steps in determination of the metastatic target organ is the recruitment of vascular endothelial growth factor receptor 1^+ (VEGFR1) bone marrow-derived haematopoietic progenitor cells (HPC)[6]. The formation of this pre-metastatic niche creates a permissive environment for incoming tumor cells[6]. After intradermal injection of either B16 melanoma cells or Lewis lung cancer (LLC) cells, $VEGFR1^+$ HPC form clusters at pre-metastatic sites (Figure 2a) that can attract the tumor cells (Figure 2b). Selective blocking of VEGFR1 (which is not expressed by tumor cells) with monoclonal antibodies completely prevented the initiation of $VEGFR1^+$ HPC clusters and formation of metastasis[6]. But how do these $VEGFR1^+$ HPC determine to migrate towards pre-metastatic organs? After injection of LLC cells, but before the formation of $VEGFR1^+$ HPC clusters, the lung pre-metastatic niche showed an increase in fibronectin expression compared to baseline levels in

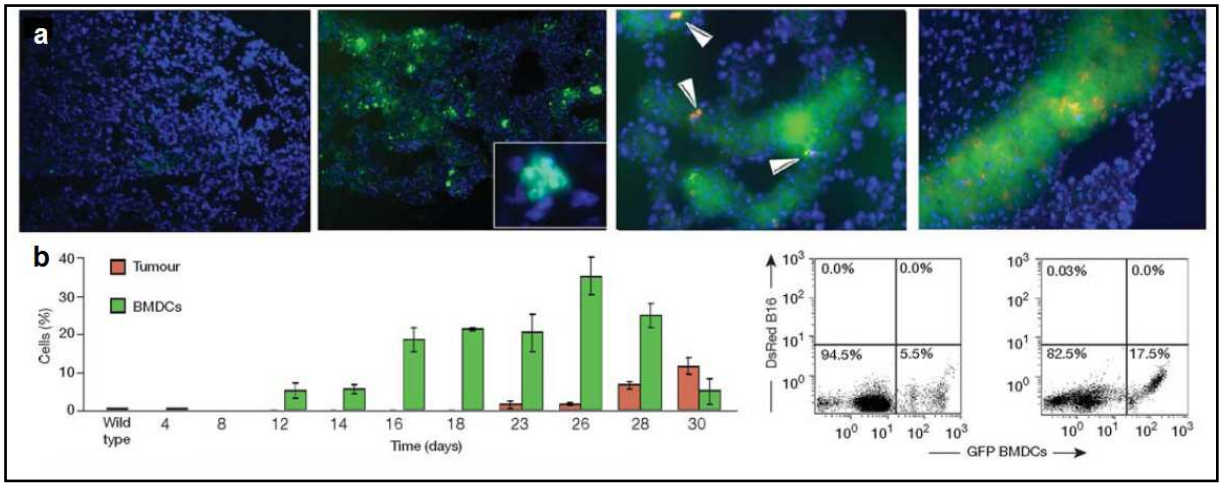

Figure 2. VEGFR1$^+$ HPCs initiate the pre-metastatic niche. (a) GFP$^+$ bone marrow cells in the lungs before implantation of DsRed-tagged B16 melanoma cells (left). On day 14, GFP$^+$ bone marrow cells are present, but no DsRed$^+$ tumor cells (left middle panel). From day 18, a few single DsRed$^+$ tumor cells start to adhere to GFP$^+$ clusters of HPCs (right middle panel). On day 23, DsRed$^+$ tumor cells proliferate at cluster sites. Cell nuclei are stained with DAPI (blue). (b) Flow cytometry analysis of cells present in the lungs. GFP$^+$ BMDCs arrive in the lungs around day 12 and their numbers peak at day 26. Around day 23, DsRed$^+$ B16 melanoma cells arrive in the lungs. Flow diagrams shows the percentage of GFP$^+$ BMDC and DsRed$^+$ B16 melanoma cells on day 14 (left) and day 18 (right). Figure adapted from [6].

wild type lung[6]. VEGFR1$^+$ HPC express VLA-4 (also known as integrin α4β1) which binds to its ligand fibronectin and mediates adhesion to the pre-metastatic niche. Moreover, binding of VLA-4 to fibronectin regulates gene expression of MMP-9 and MMP-2[59, 60]. These metalloproteinases degrade the ECM, thereby facilitating infiltration of tumor cells[31, 61]. Accordingly, MMP-9 has been shown to be a predictor of poor prognosis[33, 34]. Furthermore, clustering of VEFGR1$^+$ VLA-4$^+$ HPC together with fibronectin and fibroblasts at the pre-metastatic site resulted in an increased expression of stromal-derived factor-1 (SDF-1) in the lung parenchyma[6]. CXCR4$^+$ (which is the receptor for SDF-1) tumor cells might exploit this chemoattractant for migration and homing to distant metastatic organs[62].

Cancer stem cells

Recently, it has been proposed that cancer is a disease based on cancer stem cells. Cancer stem cells have the capacity to self-renew like normal stem cells, and therefore might drive tumorigenesis[63]. The longevity of stem cells makes it plausible to accumulate mutations and obtain a malignant phenotype[64]. Normal mammary stem cells can be isolated from the mammary gland based on phenotypic surface markers and are able to reconstitute many different cell types to form an entire functional mammary gland[65-67]. If a mammary stem cell acquires mutations that cause de-regulation of self-renewal, this could lead to development of breast cancer. However, another possibility is that cancer stem cells originate from mammary epithelial cells that have lost their differentiated state and acquire stem cell-like properties. Unfortunately, the presence of cancer stem cells makes it more difficult to design proper treatment. The quiescent state of stem cells makes them resistant to cytostatic drugs that target rapidly dividing cells. Also, stem cells express drug efflux pumps that protect the cells from drugs and cause multi drug resistance. Studies have shown that during EMT tumor cells obtain stem cell–like properties[68]. Accordingly, an invasive type of mammary epithelial cells that share stem cell-like properties has drug efflux pumps that contribute to drug resistance[69]. Survival of cancer stem cells would explain recurrence and initiation of metastasis after treatment of the primary tumor. The subject of cancer stem cell biology is a remarkable field, but in this review we will mainly focus on the potential role of cancer stem cells in the formation and initiation of the pre-metastatic niche.

Striking parallels can be found in molecular mechanisms between normal stem cells and cancer stem cells[62, 63] e.g. the regulation of stem cell trafficking. It has been postulated that the SDF-1/CXCR4 chemokine axis is a master regulator of both normal and cancer stem cell mobilization[62]. Stem cells express the G-protein–coupled transmembrane receptor CXCR4 which is the exclusive receptor for its ligand α-chemokine SDF-1 which is also known as CXCL12[62]. SDF-1 is predominantly expressed by stromal cells in the bone marrow, which during embryogenesis allows migration of haematopoietic stem cells from their site of production (liver) to the bone marrow for maturation[70, 71]. Other progenitor cells have been shown to express CXCR4 and use a SDF-1 gradient to home to the proper locations

in the body during organogenesis[62, 70]. It is not surprising that CXCR4$^+$ tumor cells (which may or may not derive from normal stem cells) use the same mechanism for developing metastases at distant sites (Figure 3). As mentioned before, tumor cells secrete a multitude of chemokines to modulate distant sites to form a permissive environment for migrating tumor cells. Several tumor types use chemokine receptors for directional migration during cancer progression[72-74]. Chemokine expression patterns were studied in several breast cancer cell lines and compared to normal mammary tissue. Both mRNA and protein levels of chemokine receptors CXCR4 and CCR7 were found to be highly expressed in breast cancer samples compared to normal mammary tissue[72]. The CXCR4 ligand SDF-1 secreted by stromal cells at the pre-metastatic organs can mediate adhesion of tumor cells to endothelium, fibronectin and stroma, which allows extravasation at specific sites[75]. Both CXCR4 and CCR7 ligands, SDF-1/CXCL12 and CCL21 respectively, were mainly expressed in lymph node, bone marrow, lung and liver which are known to be the first destinations of breast cancer metastasis[72]. Accordingly, other CXCR4$^+$ cancer types like rhabdomyosarcoma and neuroblastoma show metastatic tropism for lymph nodes and bone marrow[73, 74]. Neutralization of the SDF-1/CXCR4 axis with an anti-human CXCR4 antibody (Figure 3) resulted in a significant decrease in formation of lymph node and lung metastasis, suggesting the importance of the CXCR4/SDF-1 axis in tumor progression[72]. Remarkably, the gene encoding CXCR4 is not included in the poor prognosis signature described before[34, 52]. However, PAX genes which act as a transcriptional activator of CXCR4, have been found to be overexpressed in rhabdomyosarcoma and small cell lung cancer[76]. Also proteins involved in stress conditions, like NF-κB and hypoxia-induced HIF-1, can positively regulate CXCR4 expression[77, 78]. Tissue damage, possibly caused by adjuvant chemotherapy to destroy metastatic foci, is one form of stress which may lead to increased expression of SDF-1. As a result CXCR4$^+$ inflammatory cells like macrophages and also tissue stem cells as well as tumor cells are attracted to the site of damage for regeneration and repair.

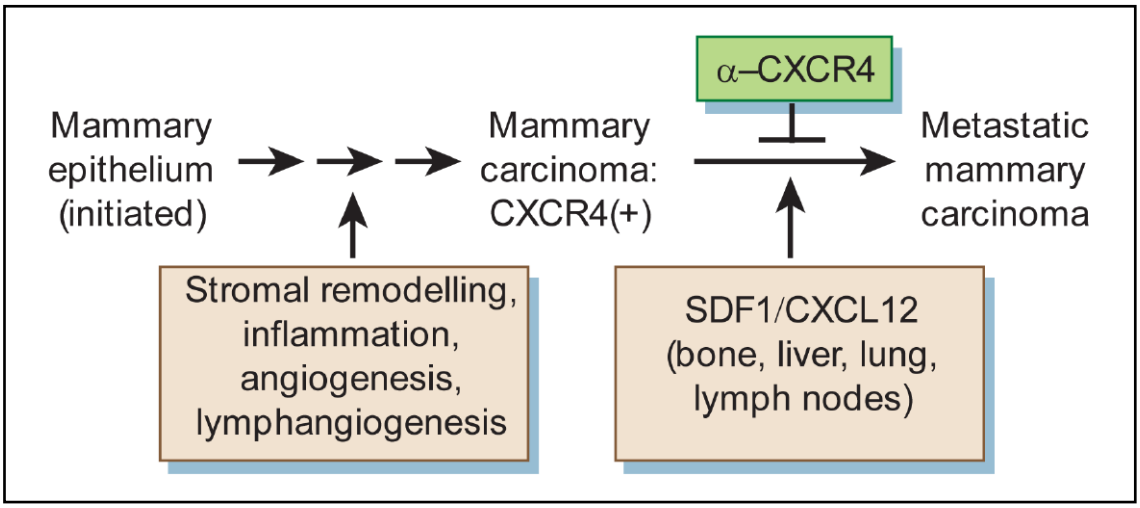

Figure 3. The role of the CXCR4/SDF-1 axis in mammary tumor progression. Mammary epithelium acquires mutations that can promote neoplastic progression. This process requires stromal remodelling, inflammation, angiogenesis and lymphangiogenesis. The transformation to mammary carcinoma often results in expression of the chemokine receptor CXCR4. Production of its ligand SDF-1 in organs like bone, liver, lung and the lymph nodes facilitates invasion and migration of carcinoma cells to secondary sites, and eventually form metastasis. Some studies have shown that blocking CXCR4 with an antibody prevents metastatic spread of tumor cells. Figure adapted from [81].

Infiltrating immune cells

For some cancer types, chronic inflammation is described as a significant cause. This was already hypothesized by Virchow in 1863, based on his observation that continuous irritation of tissue and the subsequent recruitment of inflammatory cells can cause enhanced proliferation[79]. The microenvironment of the injured tissue supports its growth and regeneration by production of proliferation-promoting factors. In this way inflammatory cells can directly increase the risk of neoplasia. Moreover, inflammatory cells can produce mutagenic compounds like oxygen and nitrogen radicals that might contribute to the accumulation of mutations in cells and eventually lead to cancer initiation[80]. In this section we aim to describe to the role of inflammatory cells recruited by the primary tumor in the formation of the pre-metastatic niche and tumor metastasis.

Tumor cells secrete a variety of growth factors like colony-stimulating factor-1 (CSF-1), granulocyte-macrophage-CSF (GM-CSF), transforming growth factor-β1 (TGF-β1) and chemokines, to recruit inflammatory cells[80]. These include neutrophils, mast cells, eosinophils, dendritic cells and lymphocytes, but the majority consists of macrophages. Macrophages have been shown to play a significant role in cancer progression and metastasis[81]. Most solid tumors contain a large population of tumor-associated macrophages (TAMs) and several studies have shown a correlation between the presence of these cells and poor prognosis in breast, prostate, ovarian and cervical cancers[82]. Therefore, it is not surprising that also CSF-1, the main growth factor for macrophages, is often overexpressed in these cancer types[80, 83] and correlates with high leukocyte density[84]. CSF-1 is a secreted chemokine that stimulates differentiation of haematopoietic stem cells into macrophages at sites of inflammation to combat pathogens. In tumors, circulating monocytes can be attracted to the site of infection and differentiate into macrophages[80]. It has been shown that CSF-1 is able to locally block maturation of dendritic cells (which are the major type of antigen-presenting cells) thereby preventing antigen presentation to cytotoxic T lymphocytes, T lymphocyte activation and killing of the tumor cells[80]. At the same time CSF-1 contributes to the development of immunosuppressive TAMs which might cause suppression of an immune response against tumor cells[80]. Primary tumors show resemblance to sites of continuous inflammation, and macrophages in this environment are 'educated' by tumor cells[80]. It is likely that a similar mechanism of immune suppression is used during tumor progression and the formation of distant metastases.

In an elegant study of Pollard and co-workers the role of TAMs in cancer progression was studied using *Csf1*^{op}/*Csf1*^{op} mice carrying a null mutation in the gene encoding CSF-1, leading to the absence of macrophages[85]. Mammary tumors were induced by expression of polyomavirus middle T oncoprotein (PyMT) under control of a murine mammary tumor virus (MMTV) LTR promoter, restricting PyMT expression to the mammary epithelium[86]. As a result, tumors develop in all mammary glands and metastatic foci form in the lungs[86]. Crossing PyMT mice with the *Csf1*^{op}/*Csf1*^{op} line gives rise to tumor-bearing mice in which CSF-1 expression was depleted. Absence of macrophages in these tumors caused a decreased rate of tumor progression[85]. Although the growth and size of primary tumors was similar, metastasis was almost completely blocked in the macrophage-deficient mice compared to wild type mice. Administration of transgenic CSF-1 in the primary tumors of mutant mice restored the phenotype by the rapid development of metastases[85]. These results suggest that CSF-1 is important for the recruitment of macrophages towards tumors and contributes to the process of metastasis.

Also other mice experiments have shown that macrophages 'educated' by primary tumor cells can increase metastatic ability[33]. Macrophages were isolated intratracheally from tumor-bearing mice and co-cultured *in vitro* with wild type lung endothelial cells. After 2 days of culture the endothelial cells expressed high levels of MMP-9 which was induced by the tumor-stimulated macrophages in a VEGFR-1 dependent mechanism[33]. Induction of MMP-9 in lung endothelial cells leads to ECM and basement membrane degradation which facilitates seeding of tumor cells in the lung. These results were confirmed by *in vivo* studies showing that tumor-stimulated macrophages promote seeding and vascularisation of tail-vein-injected tumor cells by induction of MMP-9 and VEGF in lung tissue[33].

Also macrophages themselves are able to produce growth factors and proteases that facilitate the key steps towards metastasis[80]. For example, MMP-9 secretion by macrophages leads to breakdown of the ECM and basement membrane, subsequent VEGF-A release and angiogenesis induction[55]. Macrophages can also promote angiogenesis directly by secretion of VEGF[87]. Growth factors produced by TAMs include TGF-β1, fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) and these are able to stimulate growth and the migratory ability of tumor cells[80]. Previously we described that VEGFR1^{+} HPCs arrive at the pre-metastatic niche before tumor cells do. It is not unlikely that TAMs also play a role in priming the 'soil' by inducing MMP expression in the target tissue thereby facilitating seeding of tumor cells. VEGF, either produced by TAMs or released by degradation of the basement membrane, promotes the formation of new blood vessels in the target tissue[55, 80], which is required for growth of micro- into macrometastases. Indeed, Lin *et al*. showed that the angiogenic switch was delayed in macrophage-depleted *Csf1*^{op}/*Csf1*^{op} mice, resulting in a delay in malignant transition[88]. Together, these results suggest a role for macrophages in tumor progression to malignancy.

Molecular mechanisms underlying organ-specific metastasis in breast cancer

The tissue tropism of metastasis in different types of cancer is remarkable and the underlying molecular mechanisms are still under investigation. Breast cancer shows a peculiar pattern of development of metastases in lymph, bone, lung, brain and liver. Paget suggested with his 'seed and soil' hypothesis that the formation of metastasis is determined by the interplay between circulating tumor cells and the microenvironment in the pre-metastatic organ. It is likely that in the formation of metastases, each organ orchestrates different demands on circulating tumor cells. In 1928, James Ewing challenged Paget's theory by suggesting that development of metastases in distant organs was mainly caused by anatomical and mechanical factors[38]. He proposed that the architecture of the vasculature at distant organs caused tumor cells to get trapped in capillary beds, thereby determining the sites of extravasation and formation of metastatic foci. Ectopic transplantation studies have shown that this mechanism could indeed occur at particular sites, but proliferation and growth of metastatic foci was dependent on the interaction with stromal cells in the host organ by an unknown mechanism[89, 90].

Intrinsic factors like gene-expression signatures of tumor cells can contribute to predicting metastasis to particular organs like lung[51, 54, 91] and bone[92-94]. Using highly metastatic MDA-MB-231, a cell line derived from cancer cells obtained by pleural effusion of a breast cancer patient, studies have revealed a gene-expression profile specific for marking metastasis to bone[95]. Using the organ-specific signature, researchers were able to distinguish primary breast carcinomas cell populations that will metastasize to bone and cells that would metastasize elsewhere in the body[95]. Also lung-specific expression signatures have been described[91], and expression signatures for metastasis to other organs are under investigation. In this section we will give an overview of recent studies that have contributed to unravel the molecular mechanisms underlying breast cancer metastasis to bone and lung.

Bone

The bone marrow is a well vascularised organ which may provide a favourable environment for disseminated tumor cells to survive. Stromal cells in the bone marrow microenvironment express SDF-1 which in physiological circumstances is used for retention and homing of $CXCR4^+$ HSC. However, $CXCR4^+$ tumor cells exploit this receptor axis for migration towards the bone and the formation of metastases (Figure 4). Tissue homeostasis in healthy bone can be described as a balanced interplay between osteoclasts which degrade the bone matrix, and osteoblasts which constantly create new bone matrix. The formation of bone metastases alters this homeostasis, thereby causing bone lesions. In the case of breast cancer, bone metastases often lead to osteolytic lesions (degradation of bone tissue), while bone metastases in prostate cancer often result in osteoblastic lesions (excessive formation of bone tissue)[96]. Bone-metastasized breast cancer cells induce osteoclasts to release parathyroid hormone-related peptide (PTHrP), tumor necrosis factor α (TNFα) and several interleukins, thereby causing osteolytic lesions[96]. In turn, osteoclast-secreted factors stimulate osteoblasts to secrete RANKL (ligand for receptor activator of nuclear factor-κB) which promotes differentiation of osteoclasts (Figure 4). Demineralization of bone by osteoclasts results in the release of bone matrix morphogenetic proteins like insulin-like growth factor-1 (IGF-1) and TGF-β (Figure 4). As mentioned before, these growth factors can stimulate proliferation of tumor cells and induce further expression of PTHrP. This positive feedback loop promotes constant degradation of bone matrix and results in pain and bone fractures in the patient.

Using gene expression profiling of disseminated tumor cells that show specific tissue tropism, a bone-specific signature in the MDA-MB-231 human breast cancer cell line was identified[93, 95]. Indeed, upon injection of these tumor cells into the left cardiac ventricle of immunocompromised mice, bone metastases were detectable by X-ray imaging after 10-12 weeks of inoculation[93]. The cells forming bone metastases were isolated, expanded in culture and re-inoculated into mice. By this procedure, cancer cells were selected for high bone metastatic affinity. Inoculation of these selected tumor cells resulted in rapid formation of osteolytic bone lesions in mice within 5-7 weeks[93]. Invasion of bone matrix was favoured by upregulation of a specific gene expression signature including CXCR4, MMP-1, the anchorage-molecule osteopontin, connective tissue growth factor (CTGF) and interleukin-11[93]. Indeed, upregulation of CXCR4[72, 74], IL-11 and osteopontin expression in human cancer has been correlated with the formation of metastasis in bone[93]. These results suggest that only a small fraction of a population of breast cancer cells shows specificity for the formation of osteolytic metastases in bone, and a gene expression signature is able to predict this metastatic pattern.

Lung

The lung parenchyma, like the bone marrow, is a well vascularised organ. A large network of capillaries allows the exchange of oxygen and carbon dioxide between blood and inhaled air. It has been investigated whether circulating tumor cells get trapped in the narrowing capillary beds leading to the formation of lung metastases[89, 90]. This mechanical determination can indeed occur, however, it has been shown that cancer cells migrate intentionally to the lungs by the presence of a lung-specific gene expression signature and are able to prime the 'soil' for incoming tumor cells[54]. A similar approach as described above was used to investigate gene expression specific for lung. MDA-MB-231 breast carcinoma cells were injected into the tail vein of immunodeficient mice to allow formation of metastatic lesions in the lungs. Lung metastatic cells were isolated, expanded in culture and re-inoculated into mice by tail-vain injection. The *in vivo* selected cells showed an increased lung metastatic activity compared to the parental cell line[54]. To identify a gene expression pattern based on lung metastatic activity, transcriptomic micro-arrays were performed on the highly metastatic selected cell population and the weak metastatic parental cell line. A list of genes was extracted that was distinct from the bone-metastatic gene expression signature described before, although it was derived from the same parental MDA-MB-231 cell line[93]. Several of these genes encoded secretory and receptor proteins, suggesting a possible role for the lung microenvironment providing attracting factors and contribute in seeding of tumor cells in the lung parenchyma. A subset of the genes grouped in the lung-specific signature was identified to play a role in extravasation of cancer cells at the distant site. The genes encoding epiregulin (EREG) (which can function as a ligand of EGFR), cytochrome C oxidase 2 (COX2), MMP-1 and MMP-2 are required for breaching the lung vasculature and extravasation of breast cancer cells from the circulation[53] (Figure 4). The presence of MMPs in this list of genes is in line with other studies[33, 91]. The importance of each of these genes was investigated by individual targeting and downregulation. Single knock down was not sufficient to prevent the formation of lung metastases, however, combined inhibition of all four genes abrogated the metastatic ability of the MDA-MB-231 cells almost completely[53]. The presence of a lung-specific gene expression pattern was clinically validated by studying samples from patients with primary breast cancer. Patients with cancer cells that were positive for the lung-specific signature, showed a shorter lung-metastasis-free survival compared to other patients[54].

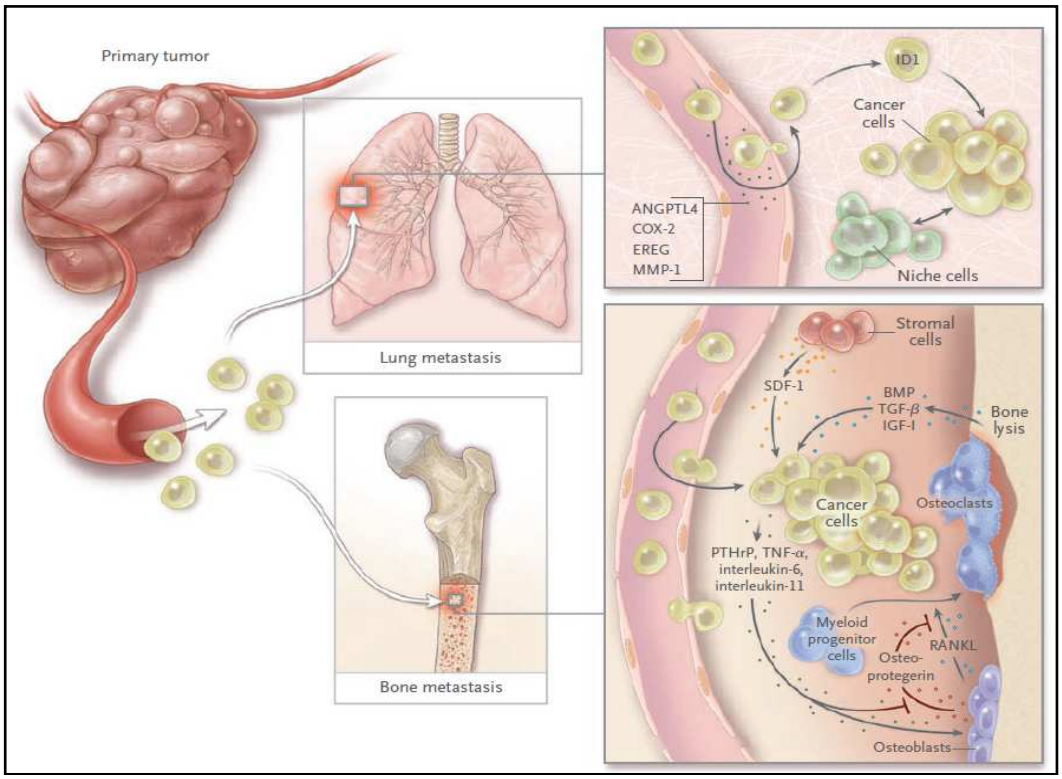

Figure 4. The molecular mechanisms underlying organ-specific metastasis in lung and bone. Organ-specific metastasis of breast cancer cells involves different genes responsible for extravasation, seeding and colonization in lung and bone. Circulating breast cancer cells expressing ANGPTL4, COX-2, EREG and MMP-1 are well equipped to extravasate the lung vasculature by breaching cell-cell junctions of endothelium (upper panels). After seeding in the lung tissue, these cells express ID1 to promote proliferation and colonization. How lung stromal cells (or niche cells) might contribute to these processes is still unknown. During bone metastasis (lower panels) tumor cells induce osteoclasts to release parathyroid hormone-related peptide (PTHrP), tumor necrosis factor α (TNFα) and several interleukins, thereby causing osteolytic lesions. In turn, osteoclast-secreted factors stimulate osteoblasts to secrete RANKL which promotes differentiation of osteoclasts. Bone lysis releases BMP, IGF-1 and TGF-β which stimulate proliferation of tumor cells. SDF-1 secreting stromal cells attract $CXCR4^+$ tumor cells. Figure adapted from [96].

Next to the intrinsic ability of tumor cells to disseminate from the primary tumor and seed in the lungs, they also contribute to the priming of the 'soil' at their preferred metastatic site by secretion of factors. Stromal cells in the breast tumor microenvironment secrete TGF-β which is able to prime cancer cells for metastasis to the lungs[97]. The ligand TGF-β binds to TGF-β receptor complexes on the cancer cell surface and thereby activates the Smad signalling pathway. Smad activates transcription of the gene encoding angiopoietin-like 4 (ANGPTL4) which enhances subsequent retention of these cancer cells in the lungs, but not in bone[97]. ANGPTL4 increases the permeability of lung capillaries by disruption cell-cell junctions in vascular endothelium, and therefore facilitates extravasation of tumor cells in the lungs (Figure 4). Experimental evidence was obtained from studying several cell lines (including MDA-MB-231) that were stimulated for several hours with TGF-β prior to tail-vein injection in mice. Compared to controls, pre-treatment of cancer cells with TGF-β resulted in an increased metastatic ability in the lungs, but not in bone[97]. Knock down of ANGPTL4 in orthotopically transplanted MDA-MB-231 cells, resulted in a decrease of dissemination of cancer cells to the lungs[97]. All together these results suggest that TGF-β secreted by the mammary stroma, promotes lung metastasis by inducing expression of ANGPTL4 in breast cancer cells.

Earlier in this review we described that VEGFR1$^+$ bone marrow-derived HPCs are the first cells that home towards the pre-metastatic lungs before tumor cells arrive[6]. Neutralization of VEGFR1 function with antibodies inhibits the formation of VEGFR1$^+$ HPC cell clusters and prevents tumor metastasis[6].

Another example of cancer cells priming the pre-metastatic lung was published by Hiratsuka *et al*. They showed that subcutaneous implanted B16 and LLC tumors secrete VEGF-A, TGF-β and TNF-α which induce the expression of chemoattracttants S100A8 and S100A9 in pre-metastatic lung tissue in tumor-bearing mice[98]. This leads to recruitment of CD11b$^+$ or macrophage antigen 1$^+$ (Mac 1$^+$) myeloid inflammatory cells, which, as described before, can cause a state of inflammation and promote metastasis. To specify the cell type that expressed these chemoattractants in the lung tissue, they sorted CD11b$^+$ myeloid cells, VEGFR2$^+$ endothelial cells and the remaining fraction of cells. Gene expression studies revealed that S100A8 and S100A9 were expressed in both endothelial cells and CD11b$^+$ myeloid cells[98]. Inhibition of S100A8 and S100A9 with blocking antibodies resulted in a reduced rate of metastases in the lungs. These results suggest that primary tumors secrete factors that modulate the pre-metastatic niche by inducing expression of chemoattractants for tumor cells as well as recruitment of inflammatory cells.

Recently, other studies have shown that CD11b$^+$ myeloid cells infiltrate into the pre-metastatic lungs of tumor-bearing mice before tumor cell arrival and promote tumor angiogenesis and metastasis[99, 100]. Breast cancer cells that are exposed to hypoxia start to express LOX[101]. Expression of LOX is associated with metastasis and poor survival in breast cancer patients[102]. In mouse models, LOX was found to accumulate in pre-metastatic lungs and crosslink collagen type IV in the ECM[100]. Consistent with previous studies, LOX accumulated at pulmonary sites with enhanced expression of fibronectin[6]. Crosslinking collagen IV leads to recruitment and adherence of CD11b$^+$ myeloid cells that were negative for the macrophage marker F4/80, suggesting that these are immature myeloid cells[100]. To validate the role of LOX in recruitment of myeloid cells, they used a model system without the presence of primary tumor cells. Tumor cells were cultured in hypoxic conditions (2% oxygen for 24 hrs) and culture media containing secreted LOX was collected. The conditioned media was injected daily in tumor-free mice for several weeks. Interestingly, the lungs of mice showed increased accumulation of CD11b$^+$ myeloid cells without the presence of tumor cells[100]. Both knock down of LOX with shRNAs in this cell line and antibody-induced inhibition of LOX activity did not induce accumulation of CD11b$^+$ myeloid cells and completely prevented the formation of lung metastasis[100]. To facilitate extravasation and invasion of bone marrow-derived cells through the basement membrane CD11b$^+$ myeloid cells produce MMP-2. MMP-2 cleaves collagen IV into peptides of which some are chemoattractants for circulating tumor cells[61]. Other evidence came from immunohistochemical analysis of human metastatic tumor samples showing that CD11b$^+$ cells were mainly found in areas stained positive for LOX, suggesting an important role for LOX and CD11b$^+$ myeloid cells in breast cancer metastasis[100].

A second study found that CD11b$^+$ myeloid cells that are also Gr-1$^+$ in the pre-metastatic lung of 4T1 tumor-bearing mice might contribute to immune suppression and tumor promotion[99]. In the pre-metastatic lung Gr-1$^+$CD11b$^+$ cells inhibit the production of interferon-γ, which is the main cytokine in the host immune response against tumors[99]. Also, these cells induce an elevated expression of inflammatory cytokines and chemokines in the pre-metastatic lung tissue compared to normal lung

tissue[99]. In addition, activation of expression of MMP-2 and MMP-9 in the lung parenchyma contributes to the recruitment of more circulating inflammatory cells and even tumor cells[99]. This positive feedback loop, together with vascular remodelling in the pre-metastatic lung, increases the probability of tumor cells to extravasate from the blood circulation and seed in this supportive microenvironment.

Subsequent to extravasation into the lung parenchyma, single cancer cells start the phase of colonization to form metastases. The establishment of metastasis requires proliferation and therefore upregulation of transcription factors regulating cell cycle progression. In triple negative breast cancer (lacking both expression of oestrogen and progesterone receptor, and human epidermal growth factor receptor 2 (HER2) amplification) a role was found for the transcription factors Inhibitor of Differentiation 1 (ID1) and 3 (ID3)[103]. Interestingly, the gene encoding ID1 was also present in the lung-specific gene expression signature described before[54]. IDs regulate cell differentiation by antagonizing the DNA binding activity of helix-loop-helix transcription factors[103]. An MDA-MB-231-derived cell line was used to study the role of ID1 and ID3 in metastatic colonization of the lungs by intravenous injection. A double knock down of ID1/ID3 in these cells resulted in a complete suppression of colonization in the lungs compared to control, suggesting that expression of these transcriptional regulators is required for proliferation and metastatic colonization in the lungs[103] (Figure 4).

Overall we can say that the molecular mechanisms underlying tumor metastasis are still not clear and more research is needed to understand the processes going on. However, many promising studies have been published recently. It is still unclear whether metastasis at distant sites might be correlated. For example, does lung metastasis only occurs after tumor cells have been recruited to the bone marrow? Do tumor cells migrate to the bone marrow, adopt features from migratory haematopoietic stem cells and continue their journey to other organs? Is there a niche-to-niche migration going on of either bone marrow-derived cells and tumor cells? How does tumor dormancy occur? What mechanism causes immune suppression in the tumor microenvironment and how can we modulate the immune system to specifically attack tumor cells? To answer these important questions on tumor metastasis we need more research on the mechanisms underlying the formation of a pre-metastatic niche.

Clinical implications

In the past decades science has made a lot of progress in understanding the process of cancer metastasis. Newly gained knowledge creates new possibilities of cancer therapeutics and patient care. In order to design new anti-metastatic therapeutics, we have to appreciate that tumor progression is the result of an interplay between intrinsic factors causing tumor cells to disseminate and extrinsic factors that favor metastasis. To inhibit cancer metastasis, targeting both the primary tumor cells and the supportive microenvironment is required. The timeframe in which tumor cells disseminate from the primary tumor is still unclear. It is assumed that this process takes place late in tumor progression, however, studies have shown that it might occur very early in tumor progression. In the clinic, by the time a primary tumor is detected, some cells might already have spread and form metastatic foci at secondary sites. Therefore, it would be more effective targeting the steps later on in tumor metastasis than targeting early steps, since it is possible that they already have occurred at the time of diagnosis. Also, metastasis is the cause of most deaths among cancer patients, so therapies that inhibit the formation of metastasis would be beneficial for patients.

Many promising studies have been focusing on predicting disease progression and clinical outcome in cancer patients. If we are able to approximate disease progression and prognosis in patients, we should use this knowledge to select the proper treatment for individual patients. The usual type of treatment of breast cancer patients is based on criteria from the National Institute of Health (NIH) and includes pathohistological grading (I-III), hormone-receptor status and axillary lymph node status[104]. Patients that are considered high risk by these criteria benefit the most from adjuvant therapy like chemotherapy or radiation after surgery[104]. Low risk patients, however, often also receive adjuvant therapy and suffer the side-effects needlessly[3]. New molecular technologies like DNA microarrays allow us to gain more insight in the interplay between the ‘seeds and soil’. In 2002, several research groups from the Netherlands Cancer Institute joined forces in a study to unravel intrinsic predictors of metastatic breast cancer. Using RNA obtained from tumor biopsies from over 100 patients with the same stage cancer, they identified a specific gene-expression signature that could predict treatment responses and overall outcome[34, 52]. In collaboration with Agendia this lead to the development of

Mammaprint, a DNA microarray that can predict survival in breast cancer patients (www.agendia.com). Mammaprint is based on a gene-expression profile of 70 genes involved in processes like cell cycle, invasion and angiogenesis, and is able to predict the interval to metastasis by placing patients into a low risk or high risk group[34]. A tumor specific gene-expression signature allows the patient to be treated accordingly. Mammaprint has been shown to be more accurate than all other clinically based predictors of the likelihood of developing distant metastases, and recent approval of the FDA of Mammaprint is a big step forward in more personalized medicine for cancer treatment.

Clinically, in cancer patients it is very hard to determine whether their disease has progressed to metastasis. This makes it difficult to set a time frame for this process and to study the distribution of metastases. Often patients do not suffer from micrometastases until they start to grow and form macrometastases. Current non-invasive diagnostic techniques like computed tomography (CT) scans and magnetic resonance imaging (MRI) are used extensively, however, their sensitivity is not sufficient to detect metastases smaller than 0.5 cm in diameter[105]. This means that once patients start to suffer from metastatic tumors, most of the times it is already too late for additional treatment. Autopsy can reveal the pattern of metastasis in an 'end-stage' manner, but autopsies are no longer part of the standard procedure in hospitals and thus many of those data are lost with the patients. The use of laboratory animals can help us in a way to simulate disease progression as it is happening in patients. With the power of new techniques like *in vivo* videomicroscopy, also known as intravital imaging, we are able to take a closer look at the metastatic process. Evidence indicates that only a few favoured cells are allowed to survive dissemination from the primary tumor, intravasate into the circularization, extravasate and home in distant organs. In that case distant metastases may be of clonal origin and different metastases can originate from different single cells[106]. These small differences in genotype and microenvironment make it more difficult for therapies to target such metastases. The identification of organ-specific gene expression signatures in cancer cells gives us more insight in the molecular mechanisms underlying metastasis, which allows us to predict the organs targeted.

To prevent homing of circulating tumor cells in target organs, the pre-metastatic microenvironment should be targeted. In principal, any gene that favors extravasation of tumor cells and primes the microenvironment is a potential target for anti-metastatic drugs. Therefore it is important to study the large number of chemokines and cytokines that are expressed by both the primary tumor and stromal cells present at the pre-metastatic site. Many of these factors a chemoattractants and inhibition may prevent tumor metastasis. For example, monoclonal antibodies against VEGFR1 prevented clustering of $VEGFR1^+$ HPCs in pre-metastatic lungs and inhibited metastasis[6]. Also tissue remodeling enzymes like MMPs might provide targets to prevent priming of the 'soil' and inhibit metastasis. Accordingly, inhibition of MMP-9 in lung tissue was able to decrease metastasis of breast cancer cells in lungs[33]. Therefore, to prevent tumor progression it might be necessary to combine therapies that target both the tumor cells and the stromal cells present at the pre-metastatic niche.

Conclusion

Breast cancer is the main cause of cancer death in women. Most deaths are caused by tumor progression and the formation of metastasis in organs like bone and lung. The process of metastasis occurs in a series of subsequent steps. Tumor cells leave the primary tumor, intravasate in the vasculature and enter the circulation. At specific sites the tumor cells extravasate and reside in a supportive microenvironment that allows colonization and the formation of metastatic foci. We gain more and more evidence that confirms that Paget's 'seed and soil' hypothesis, stating that metastasis is determined by an interplay between both tumor cells and stromal cells, is indeed true. Gene expression studies revealed that tumor cells can predict the location of metastasis and overall prognosis in patients. On the other hand, stromal cells in distant organs already create a supportive microenvironment named the 'pre-metastatic niche', before the arrival of tumor cells. Unraveling the underlying molecular mechanisms in the formation of the 'pre-metastatic niche' might contribute to the design of novel therapeutics to prevent tumor metastasis.

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