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Review

Metastasis mechanisms

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ABSTRACT

Metastasis, the spread of malignant cells from a primary tumor to distant sites, poses the biggest problem to cancer treatment and is the main cause of death of cancer patients. It occurs in a series of discrete steps, which have been modeled into a "metastatic cascade". In this review, we comprehensively describe the molecular and cellular mechanisms underlying the different steps, including Epithelial–Mesenchymal Transition (EMT), invasion, anoikis, angiogenesis, transport through vessels and outgrowth of secondary tumors. Furthermore, we implement recent findings that have broadened and challenged the classical view on the metastatic cascade, for example the establishment of a "premetastatic niche", the requirement of stem cell-like properties, the role of the tumor stroma and paracrine interactions of the tumor with cells in distant anatomical sites. A better understanding of the molecular processes underlying metastasis will conceivably present us with novel targets for therapeutic intervention.

Contents

The classical view on the metastatic cascade, starting from a primary, epithelial, neoplastic lesion includes: 1. EMT and breach of the basement membrane barrier; 2. dissociation of tumor cells from the bulk tumor; 3. invasion of the neighboring tissue; 4. intravasation into pre-existing and newly formed blood and lymph vessels; 5. transport through vessels; 6. extravasation from vessels; 7. establishment of disseminated cells (which can stay dormant for a prolonged period of time) at a secondary anatomical site; and 8. outgrowth of micrometastases and macrometastases/secondary tumors (for review, see [1–4]) (see Fig. 1 for an overview of the different steps of metastasis). Each step creates one or more physiological barriers to the spread of malignant cells. To successfully metastasize, tumor cells have to overcome all of those barriers [1,5]. In the following, the principles of metastasis are summarized, explaining at the molecular and cellular levels how tumor cells are enabled to complete each and every step of the metastatic cascade.

Recent insights have suggested yet another step, to be added at the beginning of the cascade (and therefore designated as step "0" in Fig. 1): the creation of a "premetastatic niche" at the target site, before the first tumor cells arrive at this distant location. The Lyden group showed in an elegant mouse study that bone marrow-derived cells

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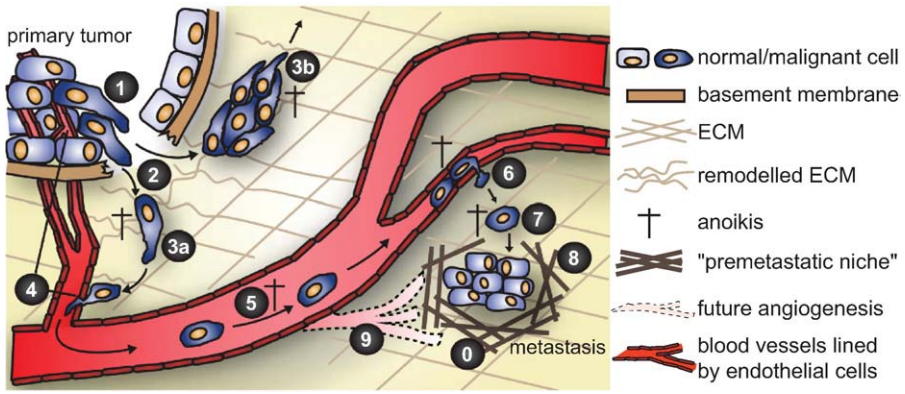

Fig. 1. The metastatic cascade. 0) Being induced by a distant tumor and mediated by bone marrow-derived cells, a "premetastatic niche" forms before metastasis becomes evident. 1) Cells in the primary tumor undergo Epithelial–Mesenchymal Transition (EMT) and acquire invasive properties. 2) Degradation of basement membranes and remodeling of the ExtraCellular Matrix (ECM) by proteinases facilitate tumor cell invasion. 3) Tumor cells invade surrounding tissue as single cells (3a) or collectively (3b). 4) Intravasation of tumor cells into newly formed vessels within or nearby the tumor. 5) Tumor cells are transported through the vasculature and arrest in a capillary bed where they extravasate (6). 7) Extravasated tumor cells can stay dormant for years. 8) Eventually, some disseminated cells grow out to a secondary tumor / macrometastasis, requiring ongoing ECM remodeling and angiogenesis (9). Cells outside their normal microenvironment undergo anoikis ("detachment-induced apoptosis"). Anoikis could hamper metastasis at several steps of the cascade, as indicated in the scheme. Not all steps of the metastatic cascade necessarily occur in a linear way. For example, premalignant tumors can already be vascularized while the timing of induction of the premetastatic niche remains elusive.

are mobilized owing to the presence of a distant, intradermal tumor. As a consequence, these cells accumulate as clusters in the lungs, where they change the local microenvironment into a niche suitable for the establishment of secondary tumors. At a later stage, tumor cells arrive at these sites and co-localize with the bone marrow-derived cell clusters [6]. The precise timing (whether at the premalignant stage or after local invasion has been initiated) of the establishment of the premetastatic niche is unknown. In line with the findings from the Lyden group, Hiratsuka and colleagues reported that distant tumors induce elevated levels of the pro-inflammatory chemokines S100A8 and S100A9 in the lungs of tumor-bearing mice [7,8]. These chemokines then attract tumor cells to the lungs via a serum amyloid A3 (SSA3)/toll-like receptor 4 (TLR4)-mediated positive feedback loop inducing NFκB. [9]. Currently, it is unclear how well these findings translate to human cancer progression. However, this could raise possibilities to therapeutically interfere with the endocrine and paracrine signaling networks required for the establishment of a premetastatic niche, thereby preventing the establishment and outgrowth of distant metastases.

1. Tumor cell dissemination and epithelial–mesenchymal transition

Epithelial tissues, representing the origins of most solid tumors, form relatively rigid sheets of cells. They are separated from the stroma by a basement membrane and are highly organized by lateral belts of cell–cell adhesion complexes. During the progression from a tumor in situ to an invasive carcinoma, epithelial tumor cells are released from their neighbors and breach the basement membrane barrier. The process underlying this phenomenon has often been suggested to involve EMT [10,11]. During EMT, initially polarized, epithelial cells acquire attributes reminiscent to those of mesenchymal cells, thereby inducing cellular invasion into neighboring tissues. Similar processes have been observed in embryogenesis, for example during gastrulation or migration of neural crest cells [12].

EMT is characterized by loss of cell polarity and downregulation of epithelial proteins, most prominently E-cadherin, but also occludin, claudins, cytokeratins or catenin proteins [10,13]. Cadherins and catenins participate in regulating cell–cell adhesion. Additionally, cells that have undergone EMT often acquire a spindle-shaped morphology, enhance cell migration and induce mesenchymal proteins like N-cadherin, vimentin, tenascin C, laminin β1 or collagen type VI α, as well as various proteinases [10,13]. The key signaling pathways and molecules inducing EMT include Receptor Tyrosine Kinases (RTKs), the transforming growth factor β (TGFB) superfamily, WNT, NOTCH, hedgehog pathway [14,15] and NFκB [16]. For example, concomitant RAS and TGFB signaling induces EMT in vitro [17] and is associated with metastasis and an EMT phenotype in a mouse skin carcinoma progression model [18]. Many of the EMT-inducing pathways play prominent roles in development and stem cell self-renewal [19,20]. Eventually, transcription factors of the snail family (SNAI1/snail, SNAI2/slug) and ZEB family (ZEB1, ZEB2), as well as TWIST1, TWIST2 and E12/E47, control the EMT transcriptome program [14,21–25]. The microRNA (miR) 200 family regulates ZEB levels, further contributing to EMT regulation [26].

EMT is likely to contain several intermediate steps, including "cell scattering" [27]. Furthermore, differences in stimuli and cell systems lead to variations in the extent of regulation of the various epithelium- and mesenchyme-specific proteins involved, as well as in the reversibility of these changes. Because of this variability and the fact that the EMT markers described in vitro await consistent confirmation in histological studies of human tumors, the role of EMT in tumor progression is still incompletely understood [28,29]. The current model proposes that EMT occurs both locally, that is, at the invasive front of tumors, and transiently, under influence of factors present in the tumor microenvironment, like TGFB [20]. Following dissemination, the reverse process, Mesenchymal–Epithelial Transition (MET), could be involved in establishing secondary tumors with an epithelial appearance, similar to the primary one [11].

EMT can promote metastasis in several ways. First, the loss of cell–cell adhesion allows for tumor cell invasion, as has been shown in E-cadherin knockout mouse models [30,31]. Invasion of tissues and vessels could be aided by a second property of cells that have undergone EMT, namely the commonly observed secretion of protein-degrading enzymes like matrix metalloproteinases (MMPs) [13]. Consistently, MMPs are often overexpressed in tumors and especially in the tumor stroma [32]. These molecules are able to remodel the ExtraCellular Matrix (ECM) within the tumor microenvironment (see below), thereby releasing and processing mitogenic and angiogenic factors sequestered by the ECM. Cleavage of ECM components like laminin 5 or collagen IV exposes cryptic sites that stimulate cell migration [33] or angiogenesis [34]. Third, E-cadherin provides not only adhesion but also functions in intracellular signaling, for example via its interacting partner δ-catenin (p120CTN). δ-catenin released from the dissolved E-cadherin complex affects the activity of small GTPases [35], corresponding to regulators of cell migration and adhesion implicated in metastasis [36]. Moreover, E-cadherin activity

can directly modulate RTK signaling [37], for instance by stimulating [38] or repressing [39] the activity of the epidermal growth factor receptor (EGFR). In addition, β-catenin signaling, a central player in the canonical WNT pathway [40] and prominently involved in many aspects of development but also in colon cancer [41], is modulated by E-cadherin [42,43]. The precise mechanism underlying this regulation remains, however, elusive. Fourth, the induction of mesenchymal proteins during EMT also promotes invasive and metastatic processes: overexpression of N-cadherin, for example, induces cell migration, invasion and metastasis [44,45]. Lastly, members of the snail and twist families of EMT mediators also inhibit apoptosis [46–49], thereby affecting both tumor growth and tumor spreading. Adding to that, snail has recently been shown to mediate tumor immunosuppression [50], potentially facilitating metastasis. Furthermore, twist blocks cellular differentiation [51–53] and can interefere with oncogene-induced senescence [54]; the putative connection between EMT, metastasis and stem cells will be discussed below.

2. Invasion and cell migration

To invade tissues and vessels, cells must acquire the ability to migrate. Extensive studies have been carried out on cells moving on 2D or within 3D matrices [55,56]. Advanced intravital microscopy technologies nowadays even allow for studying tumor cell invasion in vivo [57]. Briefly, cell migration starts with the extension of cell membrane protrusions, which is driven by a continuous cycle of actin polymerization and depolymerization. After adhesion to the ECM via integrin- and FAK-containing complexes and actin–myosin 2-mediated cell contraction, release of adhesion at the trailing edge leads to cell locomotion. In this process, the cofilin pathway acts as the "steering wheel of the cell" by coordinating membrane protrusion [58]. Cofilin and other actin cytoskeleton regulators are largely controlled by RHO family GTPases [59,60]. In line with this is the observed amplification of NEDD9 in metastatic melanoma [61]. NEDD9 is a scaffolding protein that forms part of a complex modulating RAC1 activity and cellular invasion [62]. Similarly, integrin signaling is critical for cell migration and invasion by modulating FAK/SRC signaling and the activity of RHO family GTPases [63]. In 3D ECM matrices, proteases are recruited to integrins and to other adhesion receptors on the cell surface at the leading edge, where they remodel and/or degrade the ECM to facilitate invasion [55].

Invasive tumor cells can migrate either as single cells or collectively in the form of files, clusters, or sheets. Collective invasion of tumor cells has been observed also in tumors with incomplete or no EMT. Cadherins and other cell–cell adhesion proteins thereby provide intercellular adhesion within migrating cell sheets or clusters [55,64]. Podoplanin, a small transmembrane glycoprotein, mediates collective tumor cell migration in the absence of EMT by reorganizing the actin cytoskeleton via RHOA/ROCK and ezrin [65]. A selective advantage of collective invasion of tumor cells could be that cell clones with different properties (for example, survival, migration, protease secretion) could collaborate and support each other in order to successfully metastasize. As such, a cluster of metastasizing tumor cells appears to act as one polarized entity [55].

Single cell migration can occur as slow, "mesenchymal" migration or in an "amoeboid" form, which is faster and requires no proteolytic ECM remodeling [55,66]. Many adhesion and signaling molecules, including integrins, CD44 and several Immunoglobulin-domain Cell Adhesion Molecules (IgCAMs), have been implicated in cell migration and tumor invasion [37,63,67,68]. However, their effect is often pleiotropic. For example, NCAM can prevent metastasis [69], but also promote EMT [70], possibly as a function of different isoforms and cellular context. The related IgCAM L1 is expressed at the invasive front of colon cancers [71], while both L1 [72] and carcinoembryonic (CEA) CAM1 levels [73] predict metastasis in melanoma. Another particularly interesting cell adhesion molecule is CD44: while certain splice variants induce metastasis [74], binding of osteopontin to CD44 is thought to promote homing of immune cells but also of metastatic tumor cells to various tissues [68,75]. Conversely, the standard form of CD44 has been associated with anti-metastatic functions [76].

What has become clear over time is that invasion is a plastic process and that tumor cells can adapt to different conditions by switching their properties and requirements. For example, the Friedl group showed that upon blocking protease function, cells can switch from mesenchymal to amoeboid migration in vitro and in vivo. This switch makes the cells independent of proteases and enables them to continue to invade in the presence of protease inhibitors [77]. The physical dimensions of ECM gaps and pores thereby determine the protease requirements, morphology and efficiency of cancer cell migration ([78]; P. Friedl personal communication). Intravital imaging studies in rats revealed rapid amoeboid movement of tumor cells along collagen fibers of the ECM [79]. The main difference between metastatic and non-metastatic cells in that setting was not so much the migration capacity *per se*, but rather the directionality of migration. Whereas non-metastatic mammary tumor cells moved more randomly and appeared generally unpolarized, metastatic cells had become polarized towards blood vessels and migrated more directionally [57,80,81].

3. Anoikis

As soon as tumor cells lose contact with the basement membrane during invasion they hit another barrier against metastasis: anoikis (cell death induced by inappropriate or loss of cell adhesion). Meredith and Frisch were the first to show that normal endothelial and epithelial cells actively trigger an apoptotic response once they lose their cell–cell and cell–matrix interactions or if the adhesive substrate is inadequate [82,83]. This process is thought to ensure tissue homeostasis, for example in the colon epithelium, where epithelial cells undergo apoptosis once they have reached the top of the villi [84], or during post-lactation mammary gland involution [85]. Analogous to its roles in development and homeostasis, anoikis could hamper metastasis by inducing apoptosis when tumor cells enter "foreign" environments. Conceivably, this occurs at several stages of the metastatic cascade, for example, during tissue invasion, transport through blood and lymph vessels and after extravasation at distant anatomical sites [86] (see Fig. 1). Anoikis suppression, therefore, is likely to be a prerequisite for tumor cells to successfully metastasize to distant sites [87,88]. Consistent with this, most cell lines established from human tumors contain populations of cells that survive when confronted with lack of adhesion to culture plates. Therefore, restoring anoikis sensitivity could help limiting the uncontrolled spread of metastatic tumors.

The main cell surface receptors to "sense" adhesion to the ECM and therefore to provide a cell with information about its surroundings, are the integrins [63]. Integrins are composed of heterodimers, comprising a variety of α- and β-chains. Different integrin complexes bind to diverse ECM molecules and respond by triggering an intracellular signaling cascade via focal adhesion kinase (FAK) and SRC family kinases. Furthermore, there is extensive crosstalk and signal integration between integrin and RTK signaling [89]. Therefore, it is not surprising that anoikis and integrin signaling are tightly linked [90]. Integrin activation protects cells against anoikis [83], similar to several kinases downstream of integrins, including SRC [82], FAK [91] and integrin linked kinase (ILK) [92,93]. Tumor cells often show an altered repertoire of integrin receptors [63,94] or have high levels of FAK [95], stimulating proliferation, survival and migration. Interestingly, integrin activation may also induce EMT [96], which can be achieved by downregulating E-cadherin through endocytosis [97,98], or via activation of ILK/FAK and the subsequent induction of snail [99]. Several additional studies have suggested that EMT and anoikis

suppression are linked. Blocking N-cadherin sensitizes melanoma cells to anoikis [100,101], whereas depletion of E-cadherin protects mammary cells against anoikis [30,43]. Adding to that, twist and snail are required for anoikis suppression in different cell systems [43,102]. Furthermore, the transcriptional co-repressor C-terminal binding protein 1 (CTBP1) has been shown to repress both epithelial and pro-apoptotic genes at the same time [103], providing molecular insights into the connection between EMT and anoikis suppression. Besides suppressing anoikis by interfering with cell adhesion signaling, obstruction of the apoptotic machinery may also induce anoikis suppression and facilitate metastasis: using a chick chorioallantoic membrane model, the Cheresh group showed that loss of caspase 8 in neuroblastoma cells impairs apoptosis in response to unligated integrins, which increases the metastatic potential of these cells [104].

Anoikis suppression has been used as the basis for a functional genome-wide screen for novel metastasis-associated oncogenes [105]. This led to the identification of the neurotrophic receptor TRKB, an RTK involved in the development and function of the nervous system [106]. TRKB has been reported to be overexpressed in several human malignancies, particularly those associated with metastatic activity [87]. Activated TRKB strongly suppresses anoikis in epithelial cells and endows them with oncogenic and metastatic potential [105]. Subsequent studies showed that kinase activity is critically required for the pro-oncogenic and pro-metastatic functions of TRKB, at least in the context of a model system [107], and that its signals are transferred by a twist-snail signaling axis inducing EMT [102]. This suggests that inhibition of TRKB could interfere with tumorigenesis and metastasis at several levels, making it a candidate target for anticancer therapy [108,109].

4. Angiogenesis

Tumor cell invasion alone is not sufficient to produce distant metastases; it requires also the transport of malignant cells through blood and/or lymph vessels. Pioneering work by Folkman and co-workers showed that avascular tumors cannot grow beyond a size of ~1 mm in diameter [110]. At this stage, passive diffusion of nutrients and oxygen becomes rate limiting for the tumor nodule, which is then forced to enter a state of so-called "tumor dormancy". To ensure blood supply, tumors can grow along and co-opt pre-existing blood vessels, as is seen in early stage gliomas [111]. However, in most cases, tumor vascularization is achieved by sustained (hem)angiogenesis (sprouting of new vessels from existing ones), with a significant contribution of bone marrow-derived vascular and hematopoietic progenitor cells [112]. Indeed, tumor angiogenesis is one of the hallmarks of cancer [113].

In healthy adults, angiogenesis is rare and occurs mainly during wound healing and the female reproductive cycle. Normally, the growth of new vessels is tightly held in check by a delicate balance of angiogenic activators (most prominently vascular endothelial growth factor A (VEGFA), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF)) and angiogenic inhibitors (thrombospondin 1, angiostatin, endostatin and tumstatin) [114,115]. During the "angiogenic switch", upon which a tumor activates vascularization and grows beyond its diffusion limit, this balance is tipped over to the pro-angiogenic side [116]. This is achieved through several tumor cell-intrinsic factors, but also stromal cells (especially myeloid cells) contribute prominently [117]. Once again, proteases play a crucial role, for example in the release of pro-angiogenic factors from the ECM and in the activation of angiogenic inhibitors [114,115,118]. The angiogenic switch can be made already at premalignant stages of tumorigenesis, before local invasion occurs, as is seen in several cancer mouse models and in human breast and cervical cancer [116].

Intratumor hypoxic conditions promote not only sustained angiogenesis but can also induce and select for an invasive and metastatic phenotype [119]. In this process, the hypoxia-inducible factors (HIF1A, HIF2A) play central roles [120]. Under normoxic conditions, HIF1A is targeted for degradation by the tumor suppressor VHL, an E3 ubiquitin ligase. During hypoxia, HIF1A accumulates due to increased protein stability. In tumors this can occur also by genetic alterations in oncogenes (like RAS or PI3K) or tumor suppressor genes (like VHL or PTEN). HIF1A regulates numerous target genes including many that are involved in angiogenesis (for example VEGF), cell proliferation and glucose metabolism [121]. HIF1A can promote cell migration and invasion in several ways. First, HIF1A-mediated upregulation of the CXCR4 receptor induces cell migration in Renal Cell Carcinoma (RCC) cells in vitro, while high CXCR4 levels in RCC patients correlate with poor prognosis [122]. Second, HIF1A mediates EMT by upregulating lysyl oxidase (LOX) and via activation of FAK during hypoxia [123]. Furthermore, several EMT mediators, including twist, snail, ZEB1 and ZEB2, are induced by hypoxia and HIF1A in different cancer types [124–127]. Elevated HIF1A levels in combination with twist and snail overexpression predict an increased likelihood of metastasis and reduced survival in head and neck squamous cell carcinoma [127], while increased LOX levels correlate with poor survival in breast cancer [128].

Beautiful imaging studies from McDonald and co-workers showed that the tumor vasculature is highly abnormal compared to normal blood vessels [129,130]. The normal vascular hierarchy of arterioles–capillaries–venules is changed into a chaotic organization, leading to an abnormal blood flow that changes directions or even stops locally. In combination with increased leakiness of tumor vessels, this leads to a high interstitial (tissue) pressure in solid tumors and to an inefficient supply of nutrients and oxygen. Anti-angiogenic therapeutics can induce "normalization" of the tumor vasculature, conceivably increasing the efficacy of cytotoxic agents, because of a better perfusion of the tumor [131]. In line with this hypothesis, anti-VEGF treatment significantly improves survival among chemotherapy-treated patients with metastatic colorectal cancer [132,133]. In PHD2$^{+/-}$ haplodeficient mice, tumor vessels are normalized (but not reduced in density), leading to improved perfusion of tumor xenografts [134]. As a consequence, hypoxia levels are reduced, resulting in decreased levels of metastasis-promoting genes (including those mediating EMT, see above) in tumor cells and a strong impairment of metastasis [134].

Tumor cells spread also via the lymphatic vasculature [135,136]. The presence of tumor cells in regional lymph nodes draining the primary tumor site can precede distant metastasis to visceral organs [135]. In the clinic, this is often used for prognostic purposes, for example in head and neck and breast cancer patients [137]. Most of the principles underlying tumor hemangiogenesis are conserved in lymphangiogenesis. For example, VEGF family members (VEGFC and VEGFD) induce lymphangiogenesis and lymph node metastasis via VEGF receptor 3 (VEGFR3) [138,139]. VEGFC is not induced by hypoxia [140] but rather by pro-inflammatory cytokines [141]. An open question is why tumors attract lymph vessels in the first place, as, in contrast to blood vessels, they do not provide nutrients or oxygen and thus, do not seem to confer a direct selective advantage to the tumor. One possible explanation is that lymph vessels might lower the interstitial pressure in tumors. However, many intratumor lymph vessels seem to be non-functional [142]. Another possible explanation is that lymphangiogenesis represents merely a side effect, in that blood vessel endothelial cells release growth factors like FGF2 and PDGF, which not only stimulate tumor cell proliferation but also promote lymphangiogenesis [135].

5. Intravasation–transport through vessels–extravasation

Imaging studies in living animals have recently produced detailed insight into the process of how tumor cells enter vessels (intravasation). As mentioned above, intravasation starts with tumor cells

orientating themselves towards vessels, followed by directional cell migration [80,81,143]. During the past years, the Condeelis and Pollard laboratories have established that Tumor-Associated Macrophages (TAMs) play a crucial role in this process. In xenograft and transgenic breast cancer models, macrophages were shown to "guide" tumor cells to blood vessels and sites of intravasation. This involves a paracrine signaling loop relying on the CSF1 receptor (expressed on macrophages) and EGFR (expressed on tumor cells) [144–146]. High-resolution electron microscopy showed that tumor cells protrude membrane extensions through gaps in the endothelial wall of lymph vessels [147]. Furthermore, clusters of tumor cells may enter into "leaky" lymph vessels passively, as has been observed in *Ncam* knockout/Rip1-Tag2 mouse models of pancreatic β-cell tumors [148]. Finally, tumor cells lining blood vessels and replacing endothelial cells in "mosaic vessels" or "vascular mimicry" might provide another entry site for intravasation [149].

Studies in renal cancer patients and in tumor-transplanted rats came to the conclusion that tumors may shed millions of cells into the circulation every day [150,151]. From this, it appears that intravasation is not a rate-limiting step in metastasis. However, the Condeelis group showed a direct correlation between the number of intravasated cells and the number of lung metastases in an orthotopic breast tumor rat model, suggesting that intravasation does represent a critical step of the metastatic cascade [81]. The same study showed that non-metastatic breast tumor cells were fragmented and destroyed upon entering blood vessels. Sheer forces resulting from the blood flow and lack of cellular adhesion resulting in anoikis likely eliminate disseminated tumor cells and hamper metastasis. In line with this idea is the observation that the number of circulating tumor cells in peripheral blood can be a prognostic factor in breast cancer [152,153]. The same has been shown for the presence of disseminated tumor cells in the bone marrow [154]. The bone marrow could thereby provide a transient "hiding place" for tumor cells, for example in colorectal cancer, for which tumor cells in the bone marrow are predictive for the outgrowth of metastases. However, metastases from colorectal cancer rarely form in bone and bone marrow itself [155]. It should be noted that several studies also failed to show the prognostic value of disseminated or circulating tumor cells and that the relevance of detecting these cells is much debated [156–158].

One important question arising from these observations is how long tumor cells actually circulate in the vasculature. Some studies suggest that they are trapped in the first or second capillary bed that they encounter, due to their large size relative to the diameter of capillaries [1]. Other studies claim that a large fraction of cells injected into the vasculature gets rapidly lost due to cell death [159]. In any case, it is undisputed that only a very small proportion of tumor cells entering the vasculature will eventually form a full-blown metastasis (see below).

The mechanisms underlying tumor cell extravasation from vessels into organs are likely to be similar to those contributing to invasion and intravasation. Extravasation depends on integrins [160,161] and ezrin, possibly suppressing anoikis [162]. Exposure of breast tumor cells to TGFB transiently induces angiopoietin-like 4. This multifunctional protein disrupts the integrity of blood vessel endothelium, thereby facilitating extravasation of breast tumor cells into the lung parenchyma [163]. The interaction of tumor cells with platelets, too, is important for extravasation [160,164] and anticoagulation agents can impair metastasis [165]. Integrins [160] as well as selectin proteins [166] play an important role in the interaction of tumor cells with platelets and leukocytes, but also with endothelial cells. Conceivably, formation of tumor cell aggregates facilitated by platelets protects also against anoikis and sheer stress inside the vasculature.

6. Outgrowth of secondary tumors—the "seed and soil hypothesis"

Two important observations concerning the outgrowth of disseminated tumor cells to macrometastases have been made early on: first, metastasis manifests itself not in a random pattern, but often cancers have a strong preference to spread to specific organs. Second, the outgrowth from (single) disseminated cells to micrometastases and eventually macrometastases appears to correspond to a particularly inefficient step of the metastatic cascade.

Already in 1889, the English surgeon Stephen Paget showed that breast cancer preferentially spreads to the liver, but not the spleen. Also bones form a target for breast cancer metastases, with some much more frequently colonized than others. Stomach cancer, on the other hand, rarely metastasizes to bones. These observations led to the formulation of the "seed and soil hypothesis" [3,167,168], which states that the choice of the site for a secondary tumor is made not only by the tumor cell (the "seed"), but is largely influenced also by the nature of the target organ (the "soil"). Therefore, a secondary tumor is established only if the seed can grow in the soil, that is, if the microenvironment of the target site is compatible with the properties and requirements of the disseminated tumor cell. Whereas this hypothesis was challenged for a long time [3], it is now widely accepted that the anatomic architecture of the blood flow is not sufficient to fully describe the patterns of metastatic tumor spread.

There are several, mutually non-exclusive, molecular explanations for the seed and soil hypothesis. First, the endothelia of vessels in different tissues express different adhesion molecules. Elegant in vivo phage display studies suggest that every vascular bed may have its own specific molecular "address" [169]. Tumor cells expressing the corresponding receptor can use this system to home to specific tissues. This was shown for metadherin, a tumor cell surface protein that adheres specifically to lung vessels [170]. Second, the expression of the CXCR4 receptor enables breast tumors and cell lines to form metastases in tissues that express its ligand, CXCL12. These tissues include lung, liver, lymph nodes and bone marrow, common locations for breast tumor metastases. CXCR4 stimulation induces chemotaxis in vitro, and CXCR4-blocking antibodies impair lung metastasis in SCID mouse xenograft experiments [171]. However, in vivo, CXCR4 activation could also promote the outgrowth of metastases in specific tissues, rather than invasion [172]. Third, gene-expression analysis of breast cancer cells selected for increased metastasis to either bone [173] or lungs [174] identified several genes functionally involved in tissue-specific metastasis. Translating these observations to a preclinical setting, simultaneous targeting of some of these genes (that is, EREG, MMP1, MMP2 and COX2) impairs tumor cell outgrowth in lungs [175]. Lastly, the premetastatic niche described above forms in different organs, depending on the primary tumor type. Different niche patterns can be induced with conditioned medium from tumor cells, consistent with the pattern of metastasis from those tumors [6].

Secondary tumors are thought to arise from clonal outgrowth [176]. This step is considered to be one of the most inefficient in the metastatic cascade, either because of tumor cell death or tumor cell dormancy [177]. Several studies have shown that tumor cells injected into the tail vein of mice are rapidly trapped in the lungs. However, most of these cells are subsequently cleared from the lungs [159] because of apoptosis [178], once more supporting the role of anoikis as a metastasis-suppressive mechanism. Consistent with this concept, overexpression of the antiapoptotic protein BCL2 increases lung metastasis after intravenous injection of tumor cells [178,179].

Other studies have shown that extravasated single tumor cells can reside within tissues in a state of dormancy for a prolonged period of time [180,181]. These cells remain viable and resume proliferation in vitro and form tumors upon transplantation [182]. Also in highly metastatic cells, the majority remains dormant, with only a very small proportion (~0.006%) giving rise to a large tumor burden in mice [182]. There could be several mechanisms underlying this dormancy: Folkman and co-workers showed that the size of micrometastases can remain small, due to a balance between slow proliferation and low rates of apoptosis [183]. In their xenograft model, angiogenesis is held in check by the angiogenic inhibitor angiostatin, which is secreted by

the primary tumor. When the primary tumor was surgically removed, the metastatic lesions rapidly grew out to form macrometastases [183,184]. Another important factor is the adaptive immune system, which can hold tumors and metastases in a dormant "equilibrium" state as well [185]. Interestingly, it has recently been shown that the transcription factor snail induces immunosuppression, thereby further enhancing tumor growth and metastasis in addition to its function as an inducer of EMT [50]. An elegant study from the Varmus group recently showed that the ability of (intravenously injected) cells to survive in the circulation and to establish residence in the lungs for up to 17 weeks may be present already in a subset of normal, untransformed mammary epithelial cells [186]. Subsequent activation of an inducible oncogene after seeding of these cells into the lungs was required only for their outgrowth to macrometastases, but not for their initial survival. As untransformed cells recovered from the lungs of injected mice had the potential to form mammary ducts in transplantation experiments, this suggests that some of them are multipotent stem-like cells.

A class of about a dozen "metastasis suppressor genes" has been identified [187]. When overexpressed, metastasis suppressor genes impair metastasis without affecting primary tumor growth. Several of these genes restrict the outgrowth of disseminated cells in secondary sites and act on MAPK (ERK, JNK, p38) or on RHO signaling [188]. The microRNA miR-335, too, behaves like a metastasis suppressor gene by downregulating SOX4 and tenascin C, thereby impairing cell migration and invasion [189,190].

7. Metastatic potential—where, how, why?

If metastases are clonal indeed [176], how can one cell acquire the ability to complete all steps of the metastatic cascade? Conceivably, the basic principle for metastasis is the same as for the formation of a primary tumor: tumor cell-intrinsic genetic instability facilitates the creation of a variety of cellular clones, which are challenged by Darwinian selection processes. This leads to the expansion and domination of the "fittest" tumor cell population, which can overcome all necessary barriers [4,191,192]. However, this "progression model" cannot be extrapolated fully to metastasis, as there are several theoretical and experimental objections against such a model. First, and as will be explained below, it seems that the propensity to metastasize is present in the bulk of the primary tumor and not only in a subpopulation of rare clones. Second, it is not obvious where in a primary tumor the selection pressure for metastasis comes from, unless the pro-invasive mutations would simultaneously confer a proliferative or other selective advantage [193]. Several models have been developed, aiming to explain the biological complexities of metastasis [194]. But although our understanding of the metastatic process has largely increased, none of the current models can yet fully explain the clinical and experimental observations.

Seminal experiments performed in the 1970's and 80's suggested that rare clones within a tumor cell population display a higher metastatic potential than the bulk of the tumor [176,195]. This was based on the observation that upon injection of tumor cells into nude mice, only a few metastatic clones grew out in the lungs. After several rounds of repeated injection, the cells isolated from these metastases subsequently displayed an increased succession rate to metastasize, compared to the original cells. Also when the primary tumor cell population was split into subclones prior to injection, some of these clones showed a higher metastatic potential than others [196].

However, and seemingly counterintuitive to this, recent studies showed that the gene-expression profile within a primary tumor can predict the likelihood of metastatic spread. For example, the Golub group identified 17 genes that were differentially regulated in adenocarcinoma-derived metastases, compared to a collection of unmatched primary adenocarcinomas. This "17-gene-expression signature" was found also in a subset of primary adenocarcinomas and correlated with a shorter patient survival owing to metastasis [197]. Similarly, researchers at the Netherlands Cancer Institute and from Rosetta Inpharmatics identified a "70-gene-expression signature" that predicts metastasis-free and overall survival in breast cancer patients [198,199]. This signature was generated by comparing metastatic tumor samples to a large collection of mixed, metastatic and non-metastatic, tumor samples. Together, these studies show that the tendency to metastasize is largely determined by the genes expressed in the bulk of the primary tumor [200], which seems to be in contrast to the original progression model [201].

It should be noted that the prognostic signatures cited above are not based on biological function, but instead reflect an empiric method to predict patient survival. Indeed, although several of the genes within the signatures are related to known pro-metastatic processes (including cell cycle regulation, cell invasion, angiogenesis, signal transduction and ECM remodeling), the presence of a gene in the signature does not prove its actual involvement in the pathology of the disease [202]. Furthermore, also non-malignant cells of the tumor stroma may have contributed to the signatures, depending on the relative amount of infiltrated cells in the tumor samples. In addition, not only the oncogenic mutations but also the genetic background of a patient influences the gene-expression profile of a tumor [203]. Of note, other, independently derived gene-expression signatures of breast cancer [204–207], show hardly any overlap with the 70-gene-expression signature in the identity of the genes, although the gene classes are relatively better preserved. Nonetheless, several of these gene-expression signatures predict survival equally well [208].

One possibility to reconcile these seemingly contradicting findings is to assume that a primary tumor can be metastasis-prone by harboring a large proportion of motile, invasive cells, by producing factors that induce angiogenesis or a premetastatic niche, and/or by being surrounded by an "activated tumor stroma" (see below). All of these properties would be reflected in its overall gene-expression profile. The individual tumor cell eventually growing out to form a macrometastasis, however, could still be a rare clone that, for example, must express a receptor enabling its survival and outgrowth at a distant anatomical site (or it might be a rare cancer stem cell). In such a model, the signature in the bulk tumor would predict the likelihood of distant metastasis, as the number of cells released from the tumor was shown to be proportional to the number of metastases [81]. However, a metastasis signature does not prove that the majority of cells in the primary tumor are able to complete all steps of the metastatic cascade. Consistent with this idea of merging the progression model with the prognostic gene signatures are the findings of the Massague group. Two studies describe gene-expression signatures that predict organ-specific metastasis to either bones [173] or lungs [174], which were encoded by the metastatic human breast cancer cell line MDA-MB-231. Several genes within these signatures were functionally involved in organ-specific targeting of the cells, but they showed little overlap with genes from other metastasis-predicting signatures. However, the above-mentioned 70-gene-expression signature [198] was present in the parental cell line, suggesting that within a large population of metastatic cells, subpopulations may exist that metastasize to specific organs [209]. Another example showed differences in gene-expression profiles for breast cancer cells that disseminate either via blood or lymph vessels, implying that specific genes determine which route disseminated tumor cells will take and in which location they will expand [210].

In addition to the above-described "tumor-autonomous" effects, metastasis may be influenced also in a "tumor non-autonomous" fashion, that is, by the genetic background of the host. This was revealed by studies in transgenic mice, which demonstrated that the same oncogene can cause tumors with different metastatic potential in different mouse inbred strains [211]. Strikingly, in a follow-up study [212], the expression pattern of the tumors emerging in a

"metastasis-susceptible" mouse strain, but not a "metastasis-non-susceptible" one, matched the 17-gene-expression signature predicting metastasis in solid human tumors [197]. A polymorphism in the *Sipa1* gene (a GTPase-activating protein of Rap GTPases) thereby represents one of the suspected determinants influencing metastatic progression [213]. It is conceivable that also in humans metastasis has a hereditable component [214]. This would suggest that gene-expression signatures predictive of metastasis are induced not only by tumor-associated oncogenic mutations, but also partly reflect the genetic make-up (including SNPs) of individuals. It would raise the likelihood for prospective identification of individuals carrying a higher risk of metastasis, even before the onset of cancer [215].

Another, related, question, which has remained largely unaddressed, is how metastatic cells are selected for in a primary tumor. Co-selection for pro-metastatic properties concurrently with mutations that provide growth advantage is one possible explanation. The basis for co-selection lies in the hardwiring of signaling pathways within a cell. In such a scenario, oncogenic mutations in growth factor receptors, proliferation and/or survival pathways could "take along", for example, pro-invasive pathways. Such a co-selection model has been proposed for angiogenesis [216]. The hardwiring of the various pathways would thus be cell type-specific, which could also explain why the same mutation can have different effects in different cell types.

Consistent with this idea, the cell of origin of a tumor can determine whether metastasis will occur or not. Researchers from the Weinberg group transformed different cell types (fibroblasts, mammary epithelial cells and melanocytes) with an identical set of oncogenes (that is, SV40 early region, TERT, and RASV12). As a consequence, all three cell types produced tumors in nude mice, however, only the melanocytic tumors were endowed with metastatic potential. The target tissues in which the metastases formed were the same as those seen in human melanoma patients. This suggests that the predisposition for metastasis resides within the melanocyte-specific genes (like the developmental gene and EMT-mediator slug), rather than in the oncogenic lesions [217]. Such a model is also supported by the findings of Perou et al., who classified human breast tumors into 5 different subgroups, based on their respective gene-expression profiles. Some of these subgroups express markers of different mammary cell types, which could be caused by a difference in the cell of origin [218]. The various subgroups are associated with differences in prognosis and in the propensity for metastasis [206]. However, some histopathologists have criticized this new classification and doubted its feasibility to define relevant clinicopathological entities [219,220].

8. Metastatic cancer stem cells

The model that metastases arise from cell clones, representing only a small fraction of all disseminated cells, evoked the idea that it may be metastatic "Cancer Stem Cells" (CSCs) in particular that eventually establish the macrometastases. The cancer stem cell hypothesis is receiving ever increasing attention and has been gaining experimental support in recent years [19,221]. It states that there is a hierarchical organization of cells within a tumor, similar to that in normal tissues. On top of the hierarchy stands a population of asymmetrically dividing CSCs that gives rise to rapidly expanding progenitor cells, which eventually differentiate and exhaust their proliferative potential. The stem cells retain their original phenotype and proliferative competence, which is referred to as "self-renewal" capacity. Cell populations enriched for CSCs display increased tumorigenic potential in serial transplantation assays, compared to the bulk of tumor cells. Such cancer stem cell populations have been identified first in hematologic malignancies, but subsequently also in melanoma and cancers of breast, brain, prostate, pancreatic and colon origins [221,222]. Given the similarities between normal and cancer stem cells it has been speculated that CSCs originate from self-renewing normal stem cells that have accumulated oncogenic mutations. However, it is also possible that mutations in progenitor or differentiated cells induce dedifferentiation and self-renewal capacity [19,223–225]. The self-renewal capacity and multipotency of CSCs may also be a reason for the frequently observed tumor recurrence after successful elimination of the bulk tumor, but presumably not the CSCs. As a cautionary note, the cancer stem cell hypothesis is controversial [226]. A recent publication by the Morrison group demonstrated that by optimizing the xenotransplantation procedures one can dramatically increase the frequency of tumor-initiating human melanoma cells [227]. These findings show that at least in some human cancers, tumor-initiating cells are abundant, questioning whether the cancer stem cell concept applies to all types of cancer.

To date, the link between cancer stem cells and metastasis is still largely circumstantial and based on correlations. Nonetheless, the idea that the rare metastases-forming cells correspond to the equally rare CSCs seems attractive. These cells would have the potential to form a full-blown secondary tumor, with a similar histological architecture and heterogeneity as the primary one [228]. Stem cells critically rely on the microenvironment ("the stem cell niche") they reside in. As discussed above, Kaplan et al. described such a niche being required for metastases [6]. The need to change its microenvironment into an appropriate niche, therefore, could contribute to the long latency (and often failure) of a disseminated tumor cell to grow out to a macrometastasis [229].

One link between CSCs and metastasis is the overexpression of stem cell-associated genes in metastatic tumors. For instance, the polycomb group genes EZH2 and BMI1, which function as transcriptional repressors, play a crucial role in stem cell maintenance [230] and are overexpressed in several metastatic cancers [231–234]. EZH2 levels increase with tumor progression and both a BMI1-related [235] and an EZH2-based [236] "stem cell gene signature" can predict poor survival and metastasis in cancer patients. Researchers from the groups of Chang and Weinberg showed that an embryonic stem cell gene-expression module is present in several tumor types, and is predictive for metastasis and poor survival [237,238]. Stem cell-like subpopulations isolated from lung tumor cell lines display higher in vitro invasiveness than non stem cell-like cells [239]. Hermann et al. identified CSCs in human pancreatic tumors and showed that a proportion of CSCs at the invasive front express CXCR4 [222]. Whereas all pancreatic CSCs formed tumors in nude mice, only the CXCR4-positive subpopulation metastasized. Migrating cancer stem cells have been described also for colon cancer [240], where nuclear staining of β-catenin (normally found in colon epithelium stem cells) can be detected in tumor cells at the invasive front [241]. However, the stem cell potential of these cells was not directly investigated in that study. What has been shown is that WNT signaling mediates migration and invasion of human mesenchymal stem cells [242]. Another example of stem cell-like features playing a role in metastasis is provided by the morphogen NODAL, which maintains pluripotency in human embryonic stem cells [243]. NODAL is overexpressed also in aggressive melanomas, in which it may be involved in maintaining a dedifferentiated phenotype, while it is required for the formation of tumors in nude mice [244].

Rapidly accumulating evidence suggests that a link exists between stem cells and EMT. The Weinberg group recently demonstrated that EMT induced by twist or snail endows breast epithelial cells with stem cell-like properties [245]. Conversely, normal and neoplastic stem cells isolated from breast tissues show several features of EMT. Intriguingly, several signaling pathways that mediate stem cell self-renewal (WNT, sonic hedgehog, NOTCH, bone morphogenic proteins (BMPs)) also induce EMT [14,246]. During development, neural crest cells undergo EMT and invade the surrounding tissue to reach their final destination.

These cells are progenitors of neurons and glia in the peripheral neural system, but also of melanocytes and connective tissue. The snail proteins, twist and several other transcription factors are critically involved in neural crest cell differentiation and migration. [14,247–249]. It will be interesting to investigate their contribution to cancer stem cell migration. Clearly, many aspects of tumorigenesis and metastasis are adopted from developmental processes.

9. Contribution of the microenvironment

For decades, molecular cancer biologists have studied tumor development and progression from a tumor cell-centered perspective. However, over time it has become clear that also the normal cells residing in the immediate vicinity of the tumor, the tumor stroma, play an essential role in tumorigenesis, both at early and late stages of tumor progression [250,251]. Even cells from anatomically distant tissues (for example bone marrow) influence tumor progression. The non-malignant cells in the direct surroundings of (and intermingled with) a tumor differ from those nearby the corresponding normal tissue, and create a special microenvironment [252]. The tumor stroma consists mainly of fibroblasts, ECM, vasculature and infiltrating immune and/or inflammatory cells, and has features resembling tissue undergoing wound healing. During wound healing, fibroblasts, inflammatory cells and mesenchymal stem cells infiltrate the wound and remodel the microenvironment [253]. Thereby, they orchestrate angiogenesis and cell proliferation to repair the tissue. However, whereas wound healing is a transient response, the tumor microenvironment remains in an activated state, which led to the model that tumors behave like “wounds that never heal” [254,255].

Similar to a blood clot in a wound, (pre-)malignant tumor cells secrete growth factors and cytokines, which activate and recruit fibroblasts and inflammatory cells to the tumor. These infiltrated cells, in concert with the tumor cells, further remodel the microenvironment by secreting growth factors, proteinases and ECM components [250]. This has an influence on the tumor cells but also on normal epithelial cells, on the ECM and on stromal cells. The loss of TGFB responsiveness in murine fibroblasts, for example, allows outgrowth of prostate and squamous cell carcinomas [256], implying that TGFB signaling suppresses tumorigenesis. However, in the course of tumor progression, when tumor cells become refractory to the growth-inhibitory effects of TGFB, it induces EMT and facilitates metastasis [15,257,258]. A “reactive” tumor stroma is prone to induce angiogenesis and predicts shorter survival in breast cancer [259]. In keeping with this, a gene-expression signature generated from serum-stimulated fibroblasts in vitro (mimicking a wound response) predicts increased likelihood of metastasis [204].

One of the largest components of the tumor stroma is constituted by activated fibroblasts [253]. In contrast to normal fibroblasts, these Cancer-Associated Fibroblasts (“CAFs”, also often referred to as myofibroblasts) express markers like smooth muscle actin [260]. Upon co-injection with weakly or non-tumorigenic epithelial cells, CAFs stimulate epithelial cell transformation, tumor growth, and angiogenesis [261,262]. Conversely, myoepithelial cells counteract the invasion-promoting action of CAFs in ductal breast carcinoma in situ [263]. CAFs may be derived from normal fibroblasts [264], from tumor or normal epithelial cells that have undergone EMT [265–267], or from bone marrow-derived Mesenchymal Stem Cells (MSCs), which infiltrate wounds and tumors in high numbers [268,269]. Karnoub et al. demonstrated that co-injected MSCs specifically stimulate lung metastasis in orthotopic breast tumor xenografts [269]. Activated fibroblasts have been found also at sites of liver metastasis, where they may promote the outgrowth of tumor cells [270]. Furthermore, fibroblast activation is probably involved in priming the premetastatic niche with fibronectin deposits [6] (see above). Importantly, a “humanized” stroma (through injection of activated normal human fibroblasts) is essential for the formation of ducts from human mammary epithelial cells in xenotransplantation assays [271]. Moreover, growth factor-enriched humanized stroma allows for the outgrowth of invasive carcinomas from seemingly normal human mammary epithelial cells, underscoring the requirement of a suitable microenvironment for tumor formation and progression [271].

Another crucial component of the tumor stroma corresponds to cells and mediators of the immune system, in particular the innate immune system (that is, macrophages, neutrophils and mast cells) [272,273]. Whereas the association of chronic inflammation with cancer has been made already some 150 years ago [274,275] nowadays, we have a much more profound understanding of the underlying molecular mechanisms. Inflammatory cells, CAFs alike, are recruited and activated by soluble tumor-derived factors (including colony-stimulating factor 1 (CSF1), CSF2 and TGFB) [273]. In turn, growth factors and proteinases secreted by the immune cells promote tumor growth, angiogenesis and tumor progression [273]. An adequate immune response against tumors and their antigens is suppressed, at least in part, by TGFB [276], further underscoring its ambivalent role as a tumor suppressor but also tumor progression factor [15,257]. The presence of Tumor-Associated Macrophages (TAMs) is a predictor of poor prognosis [277], promoting metastasis in breast cancer mouse models [144]. TAMs facilitate tumor cell intravasation into vessels [146] and participate in the formation of the premetastatic niche [7]. Furthermore, immune cells secreting MMP9 contribute to tumor progression in a mouse model of skin cancer [278], most likely by increasing the bioavailability of VEGF sequestered within the ECM [279]. A similar process probably takes place in glioblastoma [280].

Cells derived from the bone marrow have recently been demonstrated to be critical for systemic tumor instigation [281]. This effect was caused by tumors that secrete osteopontin (OPN) and other factors, which led to the activation and mobilization of bone marrow-derived cells. These cells subsequently infiltrated primary tumors and distant metastases where they promoted tumor expansion. Interestingly, this instigation can be provoked even by small tumors, acts systemically, and the infiltrated tumors or metastases do not need to be endowed with instigating activity themselves. As instigation enhances mainly the outgrowth, rather than the establishment, of micrometastases, it is different from the induction of the premetastatic niche discussed above [6,7]. Thus, primary tumors can have pleiotropic effects on metastasis. They can facilitate metastasis by inducing a premetastatic niche and by instigation, or they can hamper metastasis by secreting anti-angiogenic factors [184] (see above).

The complex interactions between tumor cells and host cells can be illustrated in bone metastasis. Several cancers, including those of breast, lung and prostate origins, frequently metastasize to bones [282]. In osteolytic bone metastasis, tumor cells activate osteoclasts to dissolve bone, thereby releasing growth factors that can stimulate tumor cell proliferation. Breast cancer tumor cells upregulate parathyroid hormone-related protein (PTHRP) at the site of metastasis close to bones [283]. PTHRP stimulates osteoblast cells to activate osteoclasts via the RANK receptor (TNFRSF11A). Upon bone resorption, TGFB1 and IGF1 are released and stimulate tumor cell to produce more PTHRP, constituting a vicious cycle [282]. However, RANK and RANK-ligand (RANKL/TNFSF11)-expressing cells must be in close contact with each other for stimulation, as both molecules are anchored on the cell surface. Secretion of MMP7 by osteoclasts (and potentially also by tumor cells) can increase the range of action of RANKL, by releasing it from the surface of tumor and osteoblast cells, turning it into a diffusible, active molecule [284]. RANKL-deficient neuroblastoma cells on the other hand, can trigger bone marrow-derived mesenchymal stem cells to secrete interleukin 6, which also leads to activation of osteoclasts [285]. Another bone metastasis-promoting factor is NFκB [286]. Blocking NFκB or its target gene GM-CSF (CSF2) in metastatic human mammary tumor cells impairs osteoclast formation and osteolytic bone metastases in mice [286].

Finally, it has been suggested that Loss Of Heterozygosity (LOH) of genes (including the tumor suppressors TP53 and PTEN) frequently occurs in the stroma of breast tumors and that these mutations may contribute to the epithelial-stromal crosstalk in carcinogenesis [287–291]. However, others have failed to find a significant occurrence of LOH in breast tumor stroma and argue against selection for genetic changes in stromal cells and an important role of these changes in tumorigenesis [252,292]. Because of differences in techniques used in

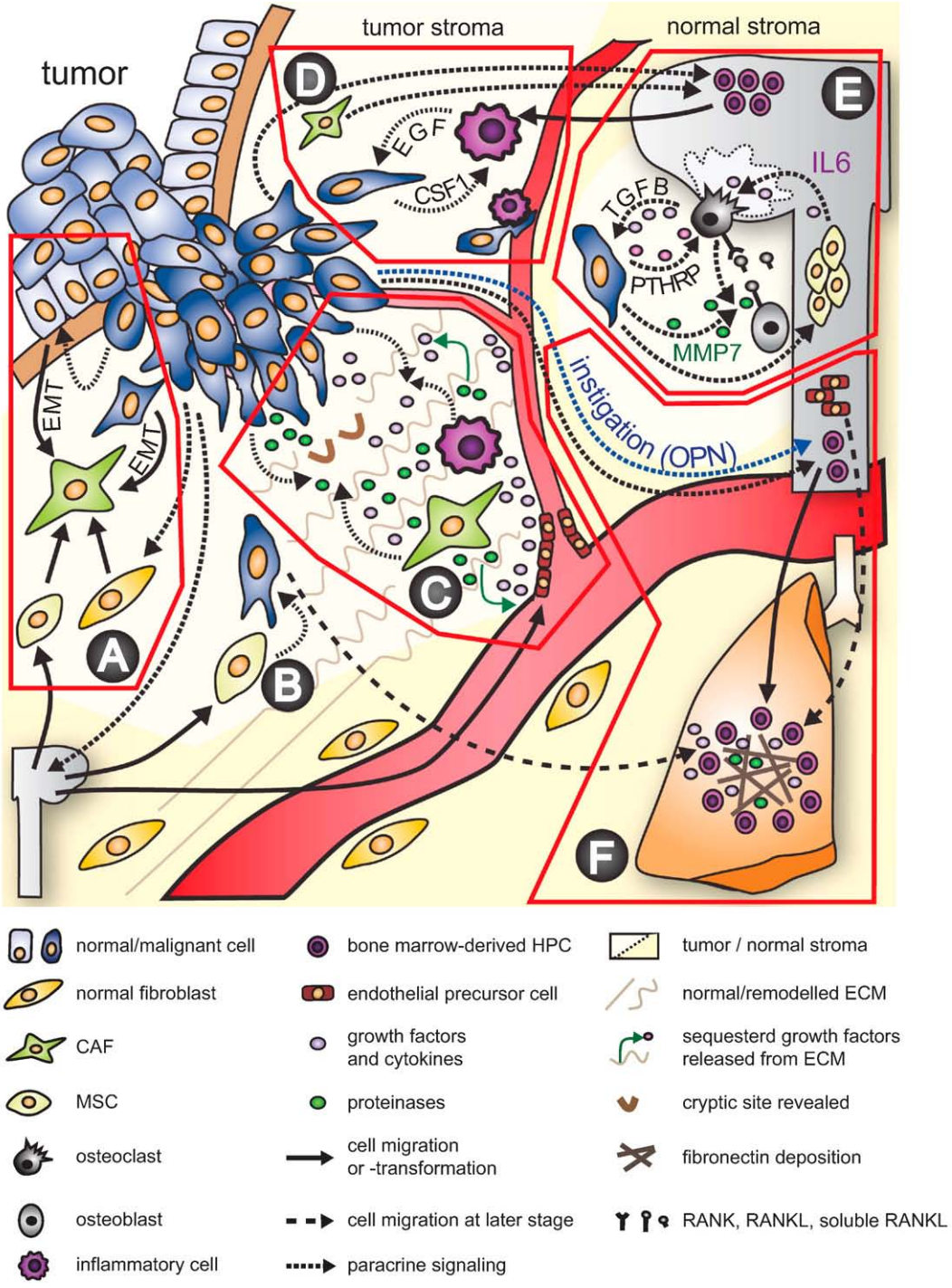

Fig. 2. Examples of metastasis-promoting functions of the tumor microenvironment. A) Origin of Cancer-Associated Fibroblasts (CAFs). Tumor cells releasing growth factors and cytokines activate normal fibroblasts or attract bone marrow-derived Mesenchymal Stem Cells (MSCs) that differentiate. CAFs can also be derived from tumor cells or normal epithelial cells that have undergone EMT. B) Mesenchymal stem cells increase the invasive potential of tumor cells. C) Tumor cells, CAFs and inflammatory cells secrete factors stimulating tumor growth, invasion and angiogenesis. Secreted proteinases remodel the ExtraCellular Matrix (ECM) and reveal pro-migratory cryptic sites or release sequestered growth and angiogenic factors. D) Hematopoietic Progenitor Cells (HPCs) are activated by a distant tumor and Tumor-Associated Macrophages (TAMs) are recruited via CSF1 signaling. TAMs attract tumor cells to vessels by stimulating EGFR and facilitate intravasation. E) The "vicious cycle of osteolytic bone metastasis" involves PTHRP secreted from tumor cells and growth factors, including TGFB, released from bone degradation by activated osteoclasts. Tumor cells can also stimulate MSCs in the bone marrow to activate osteoclasts via IL6. Release of MMP7 solubilizes RANKL, further increasing osteoclast activity. F) Formation of a "premetastatic niche" is triggered by mobilization of bone marrow-derived cells by a distant tumor. The bone marrow-derived cells infiltrate the target organ, induce fibronectin deposition and enrich the microenvironment for growth factors and proteinases. Only after the niche has been established, tumor cells infiltrate and colonize these sites. The outgrowth of established micrometastases is promoted by the activation of bone marrow-derived cells due to systemic instigation mediated by tumor-secreted OPN and other factors.

these studies, the issue of a genetic co-evolution of tumor and stroma cells requires more in-depth analysis (discussed in [252,293]).

The more cancer researchers investigate the interaction of the tumor with the host, the more complex the picture gets. What crystallizes is that these interactions occur both ways, may include the whole organism and are related to those taking place in wound healing and tissue remodeling (Fig. 2).

10. Targeting metastasis?

As metastasis is the main cause of death in cancer patients, there is a great demand for therapeutics interfering with metastasis [2,294]. However, one has to ask the question whether metastasis is "targetable" at all. Indeed, should we not focus our efforts on combating the primary tumors? At the time of diagnosis, tumor cells often have already disseminated from the primary site and can be detected, for example in the bone marrow [154]. Similar observations have been made in a mouse model for mammary epithelial hyperplasia and carcinoma in situ [295]. These disseminated cells have completed several steps of the metastatic cascade, such that invasion- and intravasation-preventing therapies would be too late at this stage. However, one might also envision that there are several rounds of seeding and re-seeding of metastases for further spread of tumor cells, for example from lymph nodes or bone marrow to visceral organs [154]. In such a setting, anti-metastatic therapies could be effective. As disseminated tumor cells and micrometastases have been found in a dormant state for a prolonged period of time [180], targeting these cells and preventing their outgrowth could be a promising approach to interfere with metastasis. However, because such cells may divide only infrequently [182], disseminated cells may be difficult to target in the absence of reliable biomarkers. Therefore, another possibility would be to interfere with the tumor microenvironment or CSC niche permissive for outgrowth of metastases, including angiogenesis. Hardly any anticancer therapies used at the moment interfere only with metastasis. Many therapeutic approaches for advanced cancers target both the primary tumor and metastases simultaneously, by blocking tumor cell proliferation and survival or tumor vascularization, which are required in both settings.

Besides the classical radio- and chemotherapies for advanced cancers, several new specific agents are being developed or have already entered the clinic. Growth factor receptors are required for tumor cell proliferation and survival, which are not only prerequisites for tumor growth, but often also for metastasis. At the same time, many of these receptors contribute also to cell migration and invasion [10]. Therefore, small molecule inhibitors and antibodies inhibiting growth factor receptors are nowadays being used to treat metastatic cancer. One promising example is the inhibition of ERBB2 in breast cancers that overexpress it [296]. ERBB2-binding antibodies increase the effect of chemotherapy on progression-free survival of patients with metastatic breast cancer [297]. They also increase patient survival in an adjuvant setting [298,299], where therapy is given after surgical removal of the primary tumor, to prevent relapse. Similarly to this antibody-based therapy, a small molecule inhibitor for ERBB1 and ERBB2 (lapatinib) also prolongs progression-free survival in combination with chemotherapy in a phase III clinical trial with patients suffering from advanced breast cancer [300].

Another approach that has been introduced recently in the clinic to reduce primary tumor and metastasis load is targeting of the angiogenic process. As angiogenesis depends largely on VEGF signaling, several strategies have been developed to disrupt this signaling route in tumors [301]. Small molecule tyrosine kinase inhibitors and antibodies against VEGF receptors can block receptor activation. VEGF-sequestering aptamers, soluble receptors and antibodies against VEGF can intercept the ligand and prevent binding to the receptor. A phase III clinical trial showed that blocking VEGF with a humanized anti-VEGFA antibody in combination with chemotherapy increases overall and progression-free survival of patients with metastatic colorectal cancer [133]. However, recent experiments in mice revealed also adverse effects of anti-angiogenic treatments [302,303]. Whereas inhibition of VEGF signaling inhibited tumor growth, it also shortened overall survival by enhancing invasion and metastasis. The mechanism underlying this phenomenon needs further investigation but it has been speculated that the induction of hypoxia or inflammation could be involved [304]. In view of these resent findings it would be interesting to test the combination of anti-angiogenic with anti-invasive therapeutics.

The most specific therapies aimed at interfering with metastasis have been developed for bone metastasis. The complex interactions of tumor cells with the microenvironment of bone metastases offer potential to intercept signaling processes required for these interactions. Bisphosphonates are molecules that can inhibit osteoclasts and several derivatives thereof are being used in the clinic to treat bone metastasis [305]. Inhibition of endothelin-1 (a growth factor for bone formation and osteoblast proliferation) could, in principle, impair osteoblastic bone metastasis. However, a phase III clinical trial failed to show an effect on disease progression in metastatic prostate cancer [306], perhaps (partly) because of a suboptimal trial design [307].

Because of the pro-invasive action of proteinases like MMPs, also MMP inhibitors have been developed and clinically tested as anticancer agents. Unfortunately, these broad range proteinase inhibitors largely failed in clinical trials [308]. The cause for this could be that several proteinases act also in a tumor-suppressive fashion, affecting tumor cell growth and survival, angiogenesis and invasion [309]. Therefore, a thorough inventory of the target (pro-oncogenic) and anti-target (anti-oncogenic) functions of the different MMPs is needed to make this protein family eligible for anticancer therapy [118].

11. Concluding remarks

Research over the last few decades has provided detailed mechanistic insight into the different steps of metastasis. The two-dimensional cell migration experiments from the 1980's and 90's have been transformed into live-imaging studies of tumor cell invasion in living animals. Whereas initially, EMT was regarded as a phenomenon primarily associated with, and relevant for, cell morphology and motility, recently a connection to (cancer) stem cells has been made. This raises the possibility that certain oncogenes endow tumor cells not only with invasive properties, but at the same time enhance their potential to self-renew. Such capabilities are likely to be crucial for a disseminated tumor cell to establish itself and grow out as a metastasis. Survival and proliferation of disseminated cells at distant anatomical sites require an appropriate niche. These niches, as well as the microenvironment of the primary tumor, are shaped and remodeled as a function of angiogenesis and under influence of a plethora of different cell types, often derived and recruited from bone marrow. This requires an extensive paracrine communication between the tumor and the host, the networks of which we are only beginning to unravel. Further progress in the understanding of the metastatic cascade will undoubtedly present us with clinically relevant clues to interfere with metastasis.

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