Published in "Seminars in Cancer Biology
doi: 10.1016/j.semcancer.2012.02.007 , 2012"
which should be cited to refer to this work.

Concepts of metastasis in flux: The stromal progression model

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The ability of tumor cells to leave a primary tumor, to disseminate through the body, and to ultimately seed new secondary tumors is universally agreed to be the basis for metastasis formation. An accurate description of the cellular and molecular mechanisms that underlie this multistep process would greatly facilitate the rational development of therapies that effectively allow metastatic disease to be controlled and treated. A number of disparate and sometimes conflicting hypotheses and models have been suggested to explain various aspects of the process, and no single concept explains the mechanism of metastasis in its entirety or encompasses all observations and experimental findings. The exciting progress made in metastasis research in recent years has refined existing ideas, as well as giving rise to new ones. In this review we survey some of the main theories that currently exist in the field, and show that significant convergence is emerging, allowing a synthesis of several models to give a more comprehensive overview of the process of metastasis. As a result we postulate a stromal progression model of metastasis. In this model, progressive modification of the tumor microenvironment is equally as important as genetic and epigenetic changes in tumor cells during primary tumor progression. Mutual regulatory interactions between stroma and tumor cells modify the stemness of the cells that drive tumor growth, in a manner that involves epithelial–mesenchymal and mesenchymal–epithelial-like transitions. Similar interactions need to be recapitulated at secondary sites for metastases to grow. Early disseminating tumor cells can progress at the secondary site in parallel to the primary tumor, both in terms of genetic changes, as well as progressive development of a metastatic stroma. Although this model brings together many ideas in the field, there remain nevertheless a number of major open questions, underscoring the need for further research to fully understand metastasis, and thereby identify new and effective ways of treating metastatic disease.

1. Introduction

Ever since metastasis has been investigated, models and concepts about how the metastatic disease process works have been suggested [1]. These have provided a framework within which to understand clinical observations and experimental findings, have served as an important tool for directing further research, and have suggested how new therapies that address metastatic disease might be developed. Most early concepts were based on clinical observations and autopsy findings. These include the "seed and soil" hypothesis that envisages tumor cells as seeds that require a particular organ microenvironment or "soil" if they are to

Abbreviations: BMDC, bone marrow-derived cell; CAF, carcinoma-associated fibroblast; CGH, comparative genomic hybridization; CSC, cancer stem cell; CTC, circulating tumor cell; DTC, disseminated tumor cells; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; FAK, focal adhesion kinase; mCSC, migrating cancer stem cell; MET, mesenchymal–epithelial transition; TAM, tumor-associated macrophage.

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survive outside of the primary tumor and grow as metastases [2], the anatomical/mechanical model that proposes that patterns of metastasis can be explained by the location of the primary tumor with respect to the blood and the lymphatic vasculature, which in turn determines to which organs disseminating tumor cells will be transported and subsequently become mechanically entrapped [3,4], and theories about metastatic cascades and generalizing sites that hold that metastases in one organ could disseminate tumor cells and give rise to metastases in further organs in a sequential manner [5,6].

The complexity of metastasis as a process determines that none of these or indeed other concepts completely and accurately describes how the process works, nor do they integrate and encompass all clinical observations and experimental findings. This can have major consequences for therapy. For example, Halstead's radical mastectomy for the treatment of breast cancer in which the axilla and its lymph nodes are removed in addition to the breast containing the primary tumor was developed on the basis of the metastatic cascade concept. The rational was that if lymph nodes containing metastatic tumor cells were left *in situ*, then these lymph node metastases could themselves give rise to metastases in other organs. Removing all lymph nodes in the axilla should therefore improve survival rates. However, large-scale long-term randomized trials have provided evidence in recent years that for a number of types of cancer removing the lymph nodes that drain primary tumors has very little effect on patient survival [7]. Furthermore, recent analysis of the growth rate of tumors suggests that within the lifetime of a cancer patient there is not enough time for the serial seeding of metastases from a metastasis elsewhere [8]. Together, these observations underline the importance of an integrated and accurate concept of how metastasis works, if efficient and effective therapies are to be developed.

In the last few years, rapid progress has been made in many areas of metastasis research. These new insights into the process of metastasis have challenged existing accepted paradigms, stimulated the development of new concepts and models, expanded our understanding of hitherto poorly understood aspects of the process, and have highlighted the need to re-evaluate and interpret existing data in the light of these new findings. In this review, we discuss long-standing concepts about how metastasis develops in the context of some of the contemporary theories that have arisen recently as a consequence of these new observations. We use the concept of the metastatic "seed" and the "soil" of the organ microenvironment – the most long-lasting and influential hypothesis in the field – as a framework within which to discuss these ideas.

2. How does the metastatic seed develop?

2.1. Clones, heterogeneity and selection

Based on a series of seminal observations in experimental animals [9,10], Fidler and others formulated the clonal selection model to explain how tumor cells acquire the ability to metastasize. This model postulates that during tumor progression, increasing genomic instability in the primary tumor results in the stochastic accumulation of genetic and epigenetic defects, resulting in a heterogeneous population of tumor cells that differ in their gene expression patterns. The gene expression profile of some of these cells will be sufficient to endow this subpopulation with the properties required for local invasion, survival in the circulatory system, extravasation into secondary organs, and growth as overt metastases at these sites. Other subpopulations of cells in the primary tumor will have some of the properties required, but will not successfully complete all the necessary steps. Thus tumor cells that successfully form metastases should be considered as "decathlon winners" [10].

In addition to experimental evidence from animal models, support for the clonal selection theory comes from histological and genetic analysis of human tumors which provides evidence for heterogeneous patterns of gene expression [11]. A corollary of the clonal selection theory is that organ-specific patterns of metastasis may be dependent on tumor-intrinsic properties that are selected for as tumor cells disseminate. Initial evidence for the existence of genes driving organ-specific metastasis came from the identification of poor prognosis gene signature through supervised clustering of cohorts of primary breast cancers [12–15]. Subsequently, gene expression signatures associated with breast cancer metastasis to bone, lung and brain were defined in experimental models and validated with human samples [16–18]. These experimental studies were based on the generation and analysis of organotropic metastatic lines derived from a parental line (mostly MDA-MB-231) by multiple rounds of *in vivo* selection. The brain and lung metastasis signature were partly overlapping and contained genes controlling vascular remodeling and permeability, such as COX2, ANGPTL4, LTBP1 and EGFR ligands. The bone metastasis signature was rather divergent, and contained genes associated with bone osteolysis and cell survival in the bone such as IL-11, PTHrP and OPN. Besides allowing the identification of individual genes, these studies proved useful for the classification of metastasis-promoting genes based on their functional contribution to metastasis. Three categories were defined: (i) metastasis-initiating genes, comprising genes that provide an advantage in tumor cell growth, escape and invasiveness at the primary tumor site; (ii) metastasis virulence genes, giving survival advantages to disseminated tumor cells within the newly colonized microenvironment; (iii) genes promoting progression, giving advantages during the entire metastatic process by affecting general steps, such as tumor angiogenesis, inflammation, epithelial–mesenchymal transition (EMT), or immune evasion. While these studies have provided unprecedented molecular details on the mechanisms of organ-specific metastasis, many questions that are relevant for the development of therapeutic strategies remain open. For example, the experimental models are based on the use of human cell lines in immunosuppressed mice, thereby bypassing a possible role of the adaptive immune system in controlling metastasis. Also, cancer cells were injected directly into the vascular system in these models, thus mimicking only the final steps of metastasis.

The clonal selection theory would not seem consistent with the observation that primary tumors are often phenotypically similar to the metastases they give rise to [19], as according to this model, metastases should represent selection of only a subpopulation in the primary tumor. Other observations, for example from gene expression profiling of primary tumors, also suggest that the clonal selection model may need to be re-evaluated [20]. These studies have defined molecular signatures in primary tumors that successfully predict patient prognosis. The majority of tumor cells in the primary tumor must express the signature for it to be detected, which does not seem to conform with the notion that a small subpopulation of tumor cells develop metastastic properties, as suggested by the clonal selection hypothesis. These data rather indicate that metastatic development is pre-defined by genetic changes acquired during the initial stages of tumor development. Consistently, transcriptome analysis suggests that primary tumors are rather similar to their matched metastases, and are more similar with each other than with tumors from other individuals [21]. Nevertheless, a number of observations make it difficult to use transcriptome analysis to draw conclusions about the provenance of the tumor cells that seed metastases with confidence, as although transcriptomically similar, primary

tumors and their matched metastases also display profound differences in their gene expression profiles [8,22]. The different genetic backgrounds of individuals may account for the more extensive differences between individuals than between their metastases and their primary tumors. Moreover, recent studies suggest that primary tumors are composed of clonal areas, which would not be detected by studies that simply take total tumor material for analysis [23]. Furthermore, the existence of a predictive 'metastatic signature' in primary tumors might not be inconsistent with the clonal selection theory, since metastatic tumor cells may self-seed back to the primary tumor and therefore 'contaminate' a primary tumor signature with a metastatic signature [24,25]. Self-seeding of the primary tumor with metastasis-derived cancer cells might also complicate the interpretation of the established relationship between primary tumor size and metastatic potential [26,27].

Variations on the clonal selection model have been proposed that help to resolve some of these issues. The clonal dominance model suggests that metastatically competent cells have a competitive advantage and therefore outgrow other subpopulations in the primary tumor [28]. The dynamic heterogeneity model suggests that the acquisition of metastatic competence may only be transient, and that the frequency with which metastatically competent cells arise determines the metastatic potential of a given tumor [29].

2.2. Is the dissemination of the metastatic seed an early or late event in the life of a tumor?

Implicit in the clonal selection theory and its variants is the idea that cancer cells need to accumulate a sufficient number of genetic and epigenetic alterations to acquire full metastatic capacity, requiring that metastasis is a rather late event during tumor progression to allow the accumulation of such alterations [30]. This notion is consistent with the generally accepted correlation between primary tumor size and risk of lymph node and distant metastasis [27], and the observation that metastatic genes are already expressed in primary tumors [31].

In the last few years a significant body of evidence has emerged which indicates that tumor cells that ultimately form metastases may disseminate very early after tumorigenesis. This notion is based on the genomic analysis of single disseminated tumor cells (DTCs), as well as matched primary tumors and their metastases from human patients [8,22]. Similarly, experimental manipulation of animal models of metastasis suggests that dissemination may occur even at pre-malignant stages of tumorigenesis [32]. Consistently, circulating tumor cells (CTCs) in the blood and DTCs in the bone marrow can both be detected at early stages of tumor development in cancer patients [30,33–37]. To accommodate these observations, an alternative model has been proposed in which tumor cells disseminate early during tumor progression, and subsequently acquire additional genetic changes that ultimately allow them to grow out as metastases at the distant site. In this model, primary tumors and metastases progress in parallel as independent lesions [8]. Clonal selection in primary tumors and metastases would be compatible with this model, but would not be a pivotal determinant of when dissemination of the metastatic seed occurs.

The comparative genomic analysis of DTCs from lymph node and bone and their corresponding primary tumors has been performed using comparative genomic hybridization (CGH) for a number of types of cancer, and provides significant evidence in support of a parallel progression model. For example, DTCs generally show fewer genetic abnormalities than their primary tumors and there is also extensive disparity between chromosomal gains and losses when DTCs and their primary tumors are compared (reviewed in [8,22]). These studies also provide evidence that genetic abnormalities in DTCs were acquired independently of those in the primary tumor, and that substantial numbers of chromosomal losses were found in primary tumors that were not present in DTCs. As loss of DNA is irreversible and transmitted to progeny, these observations provide evidence for both early dissemination of metastatic founder cells and parallel progression. However, studies on DTCs are potentially complicated by the use of epithelial markers to detect them. Tumor cells undergoing EMT, for example (see below), may not express these markers and therefore would not be included in the analysis, potentially skewing the results. Nonetheless, when matched primary breast tumors and their metastases were also compared genomically, for example using CGH, almost half of the paired samples showed more discordances than shared chromosomal abnormalities, and a substantial number of chromosomal losses were found in the primary tumors that were not present in the metastases [38]. Similar findings have been made in other studies [39,40].

In addition to this genomic analysis, other evidence also supports the notion of early dissemination and parallel progression. DTCs may remain dormant over prolonged periods of time, and a recent study demonstrated *in vivo* evolution in dormant tumor cells of the heritable ability to escape dormancy and grow out as metastases [41]. Experimentally, when untransformed mammary epithelial cells containing inducible oncogenes are injected intravenously, they can remain viable in lung tissue for prolonged periods of time before assuming malignant growth upon induction of oncogene expression [42], providing a proof of principle that even non-transformed disseminated cells have the potential to remain dormant and ultimately grow as tumors. Nevertheless, given that the definition of malignancy is the breaching of the basement membrane, it is currently difficult to envisage how tumor cells could physically disseminate at a pre-malignant stage, as has been suggested [32]. However, recent studies show that invasiveness may appear early during transformation in cells that escape oncogene-induced senescence [43], providing a mechanism for dissemination very early during tumorigenesis.

Genomic exon sequencing of colorectal [44] and pancreatic primary tumors and their matched metastases [23] revealed that the majority of point mutations were common to both primary tumors and their metastases, and that metastases had acquired a few additional mutations. This may argue against early dissemination. Indeed, these data were used to calculate when the metastastic founder cells developed, and concluded that few if any additional mutations are required for metastastic founder cells to develop from carcinomas [44], and that metastatic dissemination is a late event [23]. However, there are some important caveats associated with the interpretation of these findings. Exon analysis of protein-encoding genes was used, which by definition only addresses around 1% of the genome [45]; analysis of the genomes of primary tumors and their matched metastases on a more global level comes to different conclusions (see above). Furthermore, the analysis of point mutations in protein-encoding genes may skew the investigation toward genetic changes that underlie the tumorigenic properties of the cancer cells. Thus patterns of point mutations would be expected to be similar between primary tumors and their metastases, and these data sets may not be appropriate for making robust conclusions about the etiology of metastatic founder cells. In addition, disparity in point mutations between primary tumors and their metastases that were found in other studies support the notion of parallel progression [22].

2.3. Metastatic seeds and the pecking order: hierarchy and CSCs

Another concept for how metastasis works arises as a corollary of the cancer stem cell (CSC) hypothesis that predicts that malignancies, like many high turnover tissues, are characterized

by a hierarchical organization, with stem-like cells endowed with self-renewal and the capacity to differentiate, but also with more committed progenitor cells and fully differentiated lineages [46]. As by definition CSCs are predicted to be the cells that initiate and drive secondary tumor growth, they would be expected to underlie malignant behavior by responding to environmental cues to detach from the primary tumor and disseminate throughout the body as so-called migrating cancer stem cells (mCSCs) [19]. Thus mCSCs are predicted to be the metastatic seeds that found secondary tumors.

Experimental evidence to support the notion that CSCs play a critical role in metastasis remains thin on the ground. However, recent studies point to the existence of specific stem-like subpopulations of cancer cells endowed with high migratory and metastatic capacity, and suggest that CSCs are heterogeneous populations that include actively cycling CSCs that drive tumor growth, as well as more quiescent stem-like cancer cells. This cellular heterogeneity within the CSC compartment with the dichotomy of cycling and quiescent CSCs was first studied in pancreas cancer where the CSC population is defined by CD133 expression. The combined expression of CD133 and CXCR4, a chemokine receptor implicated in cellular migration and high malignant and metastatic potential, earmarks CTCs detectable in the portal vein which eventually form liver metastasis [47]. Accordingly, depletion of the migrating cancer stem cells using a pharmacological inhibitor of the CXCR4 receptor abrogated their metastatic potential [47]. CXCR4 expression in CSCs is likely to make them responsive to a chemotactic gradient established by its specific ligand, stromal factor 1 or SDF-1, expressed by several organs in which metastases develop.

Additional evidence for the existence of different CSCs subtypes responsible for metastasis comes from studies on colon cancer, where CSCs can be detected and prospectively enriched with a variety of cell surface antigen markers [48–52]. Three distinct types of CSCs (also referred to as tumor-initiating cells, TICs) are likely to exist in colon cancer: extensive self-renewing long-term (LT-TICs), tumor transient amplifying cells (T-TAC), and delayed contributing (DC-TICs) [53]. Only self-renewing LT-TICs were shown to be able to contribute to metastasis formation [53]. Finally, a more specific marker of migratory and distant metastasis-causing CSCs in colon cancer was recently identified: a subpopulation of $CD26^+$ cells was found in both primary and metastatic tumors from advanced stage CRC patients capable of giving rise to CTCs in the portal vein and to distant metastasis [54].

The above examples of heterogeneity in CSC populations, as well as several others [55] are likely to reflect plasticity in the CSC phenotype. Additional plasticity is also reflected in studies that show that non-CSCs can acquire CSC properties [56,57]. For example, similar to normal stem cells, a microenvironmental niche has been shown to be required to maintain glioma and skin cancer CSCs [58,59], and this is probably also the case for other tumor types [60]. A perivascular location can actually be the driving force that leads to the acquisition of CSC properties by non-CSC subpopulations [61]. Thus extrinsic microenvironmental cues are emerging as important determinants of the CSC population.

2.4. Sleeping it off: dormancy

Metastases can occur many years after surgical removal of the primary tumor, which has given rise to the concept of dormancy. These late-developing metastases are thought to develop from DTCs that have become re-activated after remaining in a stable dormant state over a prolonged period [62]. For example, after radical prostatectomy for prostate cancer, almost half of all patients have detectable DTCs in their bone marrow more than 5 years after their surgery [63]. Dormant tumor cells can exist in a quiescent state, or as micrometastases in which proliferation is balanced by cell death through apoptosis [7]. Reactivation of these dormant cells can be due to changes in the tumor cells themselves, for example due to loss of metastasis suppressor genes that regulate dormancy [64], as well as to modification of their microenvironment, for example extracellular matrix (ECM) remodeling and recruitment of inflammatory cells [65,66]. The activation of the growth of indolent tumor cells by bone marrow-derived cells (BMDC) recruited in response to osteopontin produced by a second remote "instigator" tumor may also reflect the re-animation of dormant cells [67]. Due to their quiescence or slow turnover, dormant tumor cells are resistant to conventional cytotoxic therapies because their intrinsic quiescence makes them insensitive to DNA-damaging agents that specifically target cycling cells [68].

An elegant recent study that looked at the mechanism behind the re-activation of dormant breast cancer cells in the bone marrow provides evidence that intrinsic changes in gene expression in tumor cells can relieve dormancy [41]. Metastases growing out in the bone marrow after long latency periods were found to express VCAM-1, in contrast to the parental clone that was originally injected into the experimental animals. In further rounds of injection into animals, these VCAM-1-expressing cells were able to form bone metastases without entering dormancy. Mechanistically, VCAM-1 allows breast tumor cells to recruit $\alpha4\beta1$-positive osteoclast progenitors, thereby elevating osteoclast activity that leads to bone destruction. These data nicely demonstrate that *in vivo* evolution of tumor cells can lead to the loss of dormancy.

There are a number of parallels between dormant tumor cells and CSCs. As mentioned above, CSCs can be quiescent, and are also resistant to chemotherapy. Mechanisms that CSCs share with normal stem cells underlie their innate resistance to therapy, for example multi-drug resistance due to up-regulation of cellular efflux pumps [69,70], activation of the DNA damage response [71], and lower concentrations of reactive oxygen species [72]. A perivascular location regulates CSC identity (see above), and is also required for the survival of dormant tumor cells that have disseminated to the brain [73].

2.5. Re-evaluating EMT: multiple functions in metastasis?

A concept that continues to attract attention is the notion that the morphogenetic program of EMT becomes activated in cancer cells as they progress, and that this contributes to metastasis formation. During the transition from benign adenoma to malignant carcinoma and metastasis formation, differentiated epithelial tumor cells are thought to acquire a de-differentiated, migratory, and invasive phenotype through the process of EMT [74]. This process of EMT is accompanied by dramatic changes in cellular morphology, the loss and remodeling of cell–cell and cell–matrix adhesion, and the gain of migratory and invasive capabilities [75,76]. The functional contribution of EMT to metastasis in patients is still debated, yet recent progress in the discovery of novel EMT markers provides increasing evidence for the occurrence of EMT in human cancers [19,77,78].

It is now becoming evident that EMT itself is a multistage process, involving distinct genetic and epigenetic alterations and a high degree of cellular plasticity. In the past years, a large number of genes have been identified that seem to be critical for this process [75]. A major molecular event during EMT is the loss of the epithelial cell–cell adhesion molecule E-cadherin, which by itself can suffice to induce EMT and tumor progression [79–81]. Conversely, cells undergoing EMT acquire expression of mesenchymal markers such as vimentin.

A broad-spectrum of transcriptional and post-transcriptional regulators that have been implicated in malignant progression also regulates EMT [82]. Many growth factors such as transforming growth factor β (TGFβ), and their associated signal transduction pathways induce EMT by activating one or several transcriptional

repressors, such as Snail1 (Snail), Snail2 (Slug), Zeb1 (δEF1), Zeb2 (Sip1), E47, and Twist, which in turn repress a number of genes, including E-cadherin [75,83,84]. Many other transcription factors also play critical roles in EMT [75,85]. Moreover, a number of microRNAs that are differentially expressed during EMT are pivotal regulators of the complex circuits that underlie the multiple stages of EMT [86–88]. Furthermore, several enzymatic activities and factors critical for epigenetic regulation, such as DNA methylation and histone modifications, are themselves modulated in their expression or activities during EMT [89,90]. Together, these changes orchestrate the dramatic reprogramming of cells that characterizes EMT.

Cell polarity is regulated by the Scribble, the Partitioning defective (Par) and the Crumbs complexes [91]. Loss of apical-basal polarity as a result of aberrant expression of polarity proteins is considered a prerequisite for metastatic tumor progression and leads to EMT. This is well illustrated by the Par complex that consists of the proteins Par3, Par6 and the atypical protein kinase C [91]. TGFβ downregulates Par3 expression, revealing a mechanism by which TGFβ can disrupt tight junction formation, mediate loss of apical-basal cell polarity and induce EMT [92]. Par6 of the Par complex promotes tumor initiation and progression and interacts with the TGFβ receptor. Blocking the TGFβ-dependent phosphorylation of Par6 in breast cancer models reduces metastasis to the lungs and highlights the importance of the loss of polarity signaling for EMT and metastasis [93]. Similarly, repression of the Crumbs polarity complex in epithelial tumors occurs concomitantly with increased expression of vimentin and reduced expression of E-cadherin, and its expression negatively correlates with the migratory and metastatic capacity of cells. Importantly, the proteins ZEB1 and Snail mediate repression of Crumbs, linking known regulators of EMT to polarity protein signaling through the Crumbs protein [94].

EMT appears not to be a unitary "black and white" process that leads invariably and irreversibly from a purely epithelial to a purely mesenchymal phenotype; there appear to be shades of gray in between [82,95]. It has suggested, for example, that EMT should be classified into three subtypes [95]. Furthermore, basal-like breast carcinomas often exhibit features associated with EMT, yet retain some epithelial characteristics [96]. Such intermediate states have been referred to as the metastable EMT phenotype [97]. Moreover, there is also considerable plasticity in the response to EMT induction, and is often a reversible process both physiologically and pathologically. For example, hypoxia induces a reversible EMT in breast cancer cells [98]. The reversibility of EMT in the cancer context has been used to suggest that EMT allows cells to invade and disseminate, and is then reversed at distant sites through a mesenchymal–epithelial transition (MET) that results in a metastasis that phenotypically resembles the originating primary tumor [19]. Evidence for dynamic reversible phenotypic changes *in vivo* during dissemination has been obtained for melanoma [99]. Autocrine motility factor [100] and expression of GATA3 [101] have been shown to reverse EMT. Partial EMT has been shown to decrease cell adhesion but still allow collective cell migration [102], consistent with observations that the mesenchymal (single cell) and collective modes of migration are reversibly interchangeable [103,104]. These and other such dynamic reversible changes have been suggested to be vital for dissemination [105]. The multiple levels at which EMT is regulated [82,106] provides a platform for the fine-tuning of metastable transitional states between purely epithelial and purely mesenchymal phenotypes. The spatial and temporal expression and combination of transcriptional repressors that are induced, for example, can influence the outcome of the EMT process [107]. Thus a picture emerges in which EMT describes a spectrum of phenotypes that are reversibly interchangeable and subject to dynamic regulation by the microenvironment. Dynamic interchange in the "gray scale" between purely epithelial and purely mesenchymal phenotypes as evidenced by the interplay between ZEB and miR-200 points to the importance of such transitions in tumor progression [86].

Classically, the induction of EMT has been interpreted as being important in the process of metastasis by endowing tumor cells with invasive properties. However, recent findings suggest that EMT provides many more properties of relevance to metastasis than just invasiveness. For example, EMT serves as an escape route for tumor cells from a variety of obstacles connected with cell transformation and rapid tumor growth, including oncogene addiction, oncogene-induced cellular senescence, tumor hypoxia, and increased apoptosis [43,108,109]. Apparently, EMT ensures that cancer cells not only gain migratory and invasive capabilities but also survive once they have left their accustomed primary tumor environment. Signaling pathways elicited by the EMT process provide a variety of survival signals that overcome cell cycle arrest and cell death by apoptosis or anoikis that otherwise would be triggered by the cytokine storm occurring within the primary tumor environment, by the inflammatory responses within the neighboring tissue and by the immune defense within the blood circulation. Accordingly, the genetic program of EMT includes a variety of immunosuppressive functions.

The complex changes in the cytoskeleton associated with motility and invasiveness may be incompatible with cell proliferation [110]. Accordingly, it has been shown that growth arrest can be a feature of EMT, for example through increased levels of p16ink4a [111] and repression of cyclin D expression [112,113]. Consistently, persistent expression of Twist has been associated with maintenance of dormancy and quiescence [107]. Conversely, MET is associated with increased proliferation [86].

EMT also appears to play a critical role in the generation and maintenance of cancer stem cells, consistent with the observation that many stem cell genes are expressed in metastatic cancer cells [114,115]. Indeed, EMT increases the stemness of cells, as after completion of EMT cancer cells express many stemness markers, they are able to form spheroids in culture, they are more tumorigenic in xenograft transplantation experiments and they are more metastatic [114–116]. A broad spectrum of signals from the tumor microenvironment may trigger EMT at the invasive front of epithelial malignancies, where tumor cells are in direct contact with stromal components such as fibroblasts, myofibroblasts, granulocytes, macrophages, mesenchymal stem cells, and lymphocytes that are able to secrete diffusible EMT-inducing signals [117], thereby inducing EMT, stemness properties, and facilitating detachment and dissemination from the primary site [118,119]. Moreover, quiescent stem-like cancer cells are earmarked by expression of EMT markers [75]. The ability of EMT to induce both cell cycle arrest and endow stemness properties on cells may therefore by of relevance to the quiescent CSC subpopulations mentioned above. The induction of EMT may contribute to the plasticity in the CSC phenotype, for example, endowing non-CSCs with stemness properties. However, the degree to which genetic programs that regulate stemness and EMT overlap remains to be properly investigated. EMT has also been suggested to generate mCSCs that leave the primary tumor and disseminate to distant sites, subsequently undergoing MET to resume growth and form metastases that are phenotypically similar to the primary tumor from which they are derived [19,86].

Finally, cells that have undergone EMT are found to exhibit increased resistance against many, but not all chemotherapeutic agents [116]. Interestingly, the converse is also true: chemical entities have been found that eradicate with higher efficacy cells that have undergone EMT as compared to their epithelial counterparts, raising the possibility of directly targeting cells that have undergone EMT [120].

3. Concepts of the soil

The last few years have seen a dramatic increase in our knowledge about key constituents of the microenvironmental "soil" that supports the survival and outgrowth of the metastatic "seed" in distant organs. It has become clear that the microenvironment around DTCs has a profound influence on whether they die, remain dormant or grow as metastases [7]. Different tumor types may have different microenvironmental requirements for metastatic outgrowth. Such differences may contribute to differences in intrinsic metastatic potential, namely the tendency for some tumor types (*e.g.* melanomas) to form metastases even when the primary tumor is very small, while other tumor types (*e.g.* basal cell carcinomas) rarely metastasize even after sizable growth of the primary tumor [6]. Similarly, particular microenvironmental requirements for the survival and growth of DTCs from different types of cancer may underlie organ-specific patterns of metastasis.

3.1. Inflaming the situation: a niche occupation

A microenvironment that is conducive to the growth of DTCs has been termed a metastatic niche [121]. Recent years have seen a number of seminal studies that have identified key constituents of such niches. Remodeling of the ECM and recruitment of inflammatory cells and other BMDC play a central role [122–125]. Growth factor, cytokines, chemokines and other proteins produced by cellular components of the metastatic niche are pivotal in the formation of metastatic niches, for the attraction of CTCs, and for the survival and outgrowth of DTCs [122–124,126]. A number of observations also suggest that a perivascular location is a pre-requisite for DTC survival and outgrowth [73], and there is increasing evidence that hypoxia plays an important role in the metastasis-promoting function of metastatic niches [126–128]. Progressive changes in the stroma of primary tumors takes place during tumor formation and progression [129,130], and there are also many similarities between these changes and the constituents of metastatic niches.

Metastatic niches may be found endogenously in organs where metastases form. A higher prevalence of such niches may underlie the predilection of DTCs to grow as metastases in organs such as lymph nodes, lungs, liver, brain and bone. A number of observations suggest that by occupying the normal stem cell niche, for example in the bone marrow, DTCs find a primed niche that supports their growth [131,132]. Nevertheless, endogenous metastatic niches are probably sparsely distributed, which may account in part for the inefficiency of the metastatic process. For example, injection of tens of thousands of tumor cells intravenously only generates several hundred metastases, even after several rounds of selection for the ability to grow as experimental metastases in the lungs after intravenous injection which would be predicted to highly enrich for cells with metastasis-forming ability [133].

Remodeling of the organ microenvironment has been demonstrated in recent years to create metastatic niches that foster the outgrowth of DTCs. These niches can be induced by primary tumors prior to the settling of DTCs in organs – so-called pre-metastatic niches – that can also attract CTCs through growth factors, cytokines and other chemoattractants that are produced by niche components [122–124]. In experimental models, pre-metastatic niche formation has been shown to be critical for the formation of fulminant metastases [122–124]. Formation of metastatic niches after removal of the primary tumor, for example due to inflammatory processes, may be responsible for the re-activation of dormant DTCs, although experimental evidence to support this notion still remains to be garnered.

It is notable that many of the components of metastatic niches and their formation are related to inflammatory processes. Pro-inflammatory members of the S100 family and members of the Serum Amyloid A acute phase proteins have been identified as having a pivotal role in the formation and function of metastatic niches, playing a key role, for example, in the recruitment of CD11b+ myeloid cells to these sites [134]. Monocyte and macrophage-specific chemokines are also expressed [123]. The remodeling of the ECM (see below), such as the production and deposition of fibronectin and the activities of lysyl oxidases and proteases, is a hallmark of both sites of inflammation and of pre-metastatic niches [135]. Hypoxia, an emerging niche feature that also induces expression of lysyl oxidases, can also promote inflammatory responses [136]. In addition to releasing cells from dormancy in the bone [41], VCAM-1 expression on tumor cells has also been recently shown to mediate their interaction with metastasis-associated macrophages, providing a survival advantage [137]. Taken together, these observations suggest that the formation of metastatic niches recapitulates the inflammatory processes and tumor–stroma interactions that drive primary tumor growth, and thereby fosters metastasis formation by DTCs.

3.2. Digging over the soil

Remodeling of the ECM has emerged as an important event during the establishment of metastatic niches. MMP-9, produced for example by VEGFR1+ BMDC, is required for the formation of pre-metastatic niches and the outgrowth of secondary tumors in the lung [122,138]. Additional ECM components such as fibronectin [122], periostin [139] and tenascin-C [140] are produced in these niches, and existing ECM components are modified, for example through the activity of lysyl oxidases, enzymes that cross-link collagen and elastin [126]. Together, these and other mechanisms serve to modify the ECM, thereby creating a microenvironment that is permissive for the growth of DTCs.

ECM remodeling may act in a number of ways to promote the outgrowth of metastases. Changes in the constituents of the ECM can of course serve to modify epitopes with which integrins and other receptors on the surface of tumor cells can interact. Integrin-mediated activation of focal adhesion kinase (FAK) signaling promotes cell survival and proliferation [141] and can regulate CSC properties [142]. Remodeling of the ECM can also be sufficient to re-activate dormant tumor cells, for example mediated by integrin-FAK signaling [65,143]. Induction of periostin expression by fibroblasts in metastatic niches is required for recruitment of Wnt ligands and the maintenance of CSC properties in DTCs [139]. Evidence is also emerging that an important outcome of matrix remodeling is an increase in the stiffness or rigidity of the microenvironment in a manner that can have a profound effect on cell behavior. For example, matrix cross-linking mediated by the activity of lysyl oxidases increases focal adhesion formation and FAK activation, and promotes invasiveness and malignancy [144]. Caveolin1 expression on carcinoma-associated fibroblasts (CAFs) remodels and stiffens the ECM microenvironment, and consequently promotes metastasis formation [145]. Matrix stiffness also regulates the activity of the TAZ transcription co-activator that forms part of the Hippo pathway [146], and TAZ activity confers stemness properties on breast cancer cells [147]. In hepatocellular carcinoma cells, stiffer matrices were found to promote proliferation and chemoresistance, while cells surviving after chemotherapy on softer matrices exhibited a reversible dormant phenotype associated with expression of CSC markers [148]. Finally, increased matrix stiffness favors TGFβ-induced EMT over apoptosis [149]. Thus a picture emerges in which enhanced matrix stiffness maintains or endows CSCs properties on tumor cells, can regulate dormancy, and determines the response to EMT-inducing factors.

3.3. Long range fertilizers of the soil

A remarkable finding that has emerged from the study of the formation of pre-metastatic niches is the long-range signaling that allows primary tumors to establish metastatic niche structures. Factors such as VEGF-A and PlGF produced by primary tumors act distantly on the bone marrow to mobilize VEGFR1+ BMDC that contribute to pre-metastatic niche formation [122]. Similarly, primary tumor-derived VEGF-A, TNFα and TGFβ induce expression of S100A8 and S100A9 in developing pre-metastatic niches, which in turn recruits CD11b+ myeloid cells [123].

Recent studies have implicated primary tumor-derived microvesicles and exosomes in the long-range signaling involved in pre-metastatic niche formation [150]. Microvesicles and exosomes contain membrane and cytoplasmic proteins, as well as nucleic acids derived from the cell of origin. They can be transported *via* the blood, and the cargo they carry can interact with target cells and modify their behavior [151]. Exosomes released from rat pancreatic adenocarcinoma cells together with CD44v6 in the soluble fraction complement each other in generating a niche for efficient tumor outgrowth [152]. Microvesicles released from CD105-positive renal carcinoma CSCs stimulate angiogenesis, upregulate VEGF-A, MMP2 and MMP9 expression in pre-metastatic sites in the lung, and promote lung metastasis [153]. Microvesicles have also been shown to be involved in the bilateral communication between tumor cells and fibroblasts, with tumor-derived microvesicles acting to upregulate MMP9 expression in fibroblasts [154].

The requirement for long-range signals derived from primary tumors that orchestrate the formation of pre-metastatic niches may account for the association between elevated risk of metastasis development and increasing primary tumor size. It would seem reasonable to assume that the tumor-derived growth factors and other signaling molecules involved would need to rise above a given systemic concentration threshold before having an effect in the bone marrow or potential sites of pre-metastatic niches. Larger tumors would be expected to produce more of the requisite signaling molecules, and therefore the concentration of these molecules in the circulatory system should also rise concomitantly. Thus a niche environment that supports the outgrowth of DTCs as metastases may only develop once the primary tumor reaches a sufficient size to produce enough signaling molecules to activate niche formation.

4. Putting it all together: conflicting or compatible concepts?

As outlined above, a number of novel concepts have arisen recently as a result of new groundbreaking experiments, and existing concepts have also been modified as a result. These concepts often only consider one particular aspect of metastasis, and none of them completely explain the process, nor account for all experimental findings. Is it possible to synthesize a concept on the basis of the data that has been generated to date that unifies these different concepts and provides a more comprehensive overview of the process of metastasis? Some of the concepts above are apparently conflicting, for example regarding the question of whether the metastatic dissemination that ultimately gives rise to metastasis is an early event after tumorigenesis or rather occurs late in tumor development. It is possible that no single concept explains the process of metastasis, and that the mechanisms differ between cancer types or even between individual patients. Nevertheless, the process of metastasis is comparable for many different types of cancer (local progression and invasion, transport in the circulatory system, extravasation, survival and growth at (often similar) secondary sites), suggesting that common mechanisms are probably operative. Furthermore, there are considerable similarities between several of the concepts outlined above, which provide a foundation for putting together the pieces of the metastasis concept jigsaw puzzle.

Striking areas of convergence are the commonalities that have emerged between the regulation of EMT, stemness, dormancy and therapy resistance. Many of these are pointed out above. The similarities between CSCs and cells that have undergone EMT have been recently extensively reviewed [110,116]. A further example is provided by CXCR4. In addition to marking CSCs that will form metastases, CXCR4 and its ligand SDF-1 have been implicated in regulating EMT in breast cancer [155], oral SCC [156] and pancreatic cancer cells [157], and probably act in conjunction with TGFβ [158,159]. Similarly, CXCR4 is associated with chemoresistance [160] and reversible dormancy [148].

It is also striking that many of the constituents that have been described as being crucial for metastatic niche function serve to regulate EMT, stemness, dormancy and therapy resistance. For example, VEGF-A drives the formation of pre-metastatic niches [122], creates a perivascular niche that maintains the stemness of skin tumor CSCs [59] and suppresses dormancy [73]. EMT is induced by inflammatory regulators that are present in metastatic niches [161], as exemplified by IL-1β in head and neck cancer [162]. The ECM remodeling that typically occurs in inflammation and fibrosis is very similar to that found in metastatic niches, and contributes to EMT [95]. Consistently, the lysyl oxidase LOXL2 induces EMT *via* increasing the stability and activity of Snail1 [163]. MMPs are also activated in the metastatic niche and induce EMT [164]. The metastatic niche constituent periostin regulates CSC properties, as well as EMT [165]. Hypoxia promotes CSC stemness, as well as the formation of a CSC niche [166]. Furthermore, hypoxia is also a potent and reversible inducer of EMT [98], and a recent study implicates it in inducing dormancy in glioblastoma CSCs [167].

5. The stromal progression model

The above observations indicate that there is a tight interconnection between EMT, stemness, dormancy and therapy resistance, and it is likely that the metastatic niche plays a critical role in regulating these processes at sites where secondary tumors develop. These and the other observations described above allow us to tentatively suggest a concept of metastasis that we have called the stromal progression model (Fig. 1). The tumor stroma is comprised of ECM, non-malignant cells and the signaling molecules they produce. In the stromal progression model, progressive co-evolution of the tumor stroma and the genetic make-up of tumor cells at both the primary and secondary sites provide the platform required for metastasis formation. This model accommodates many aspects of the disparate models and theories that have been suggested to date, and is outlined in detail in the following text.

Similar to clonal selection models, the stromal progression model suggests that serial acquisition of genetic mutations and aberrations driven by increasing genomic instability occurs in tumor cells during primary tumor progression, together with epigenetic changes. However, stromal progression also occurs in parallel, for example the progressive remodeling of the ECM in the tumor, activation and recruitment of stromal cells such as fibroblasts and BMDC, regional hypoxia, the induction of angiogenesis and the development of an inflammatory milieu. Breach of the basement membrane and subsequent invasion further exposes tumor cells to new microenvironments and further stimulates stromal progression. Thus the dynamic stepwise mutual and interdependent cross-regulation between tumor and stromal cells leads to progression of the tumor as a whole. In the absence of an appropriate

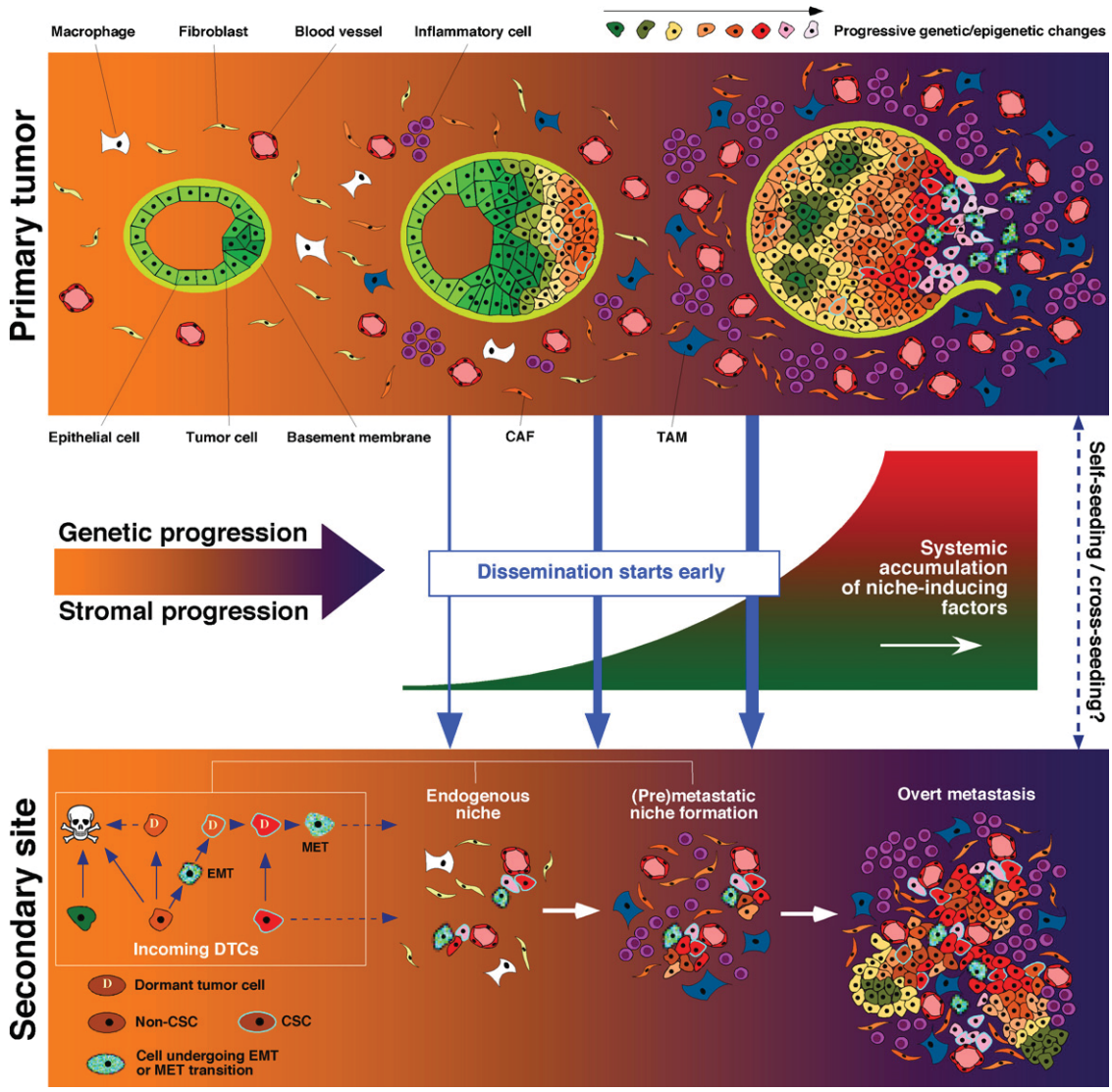

Fig. 1. *The stromal progression model.* During the development of the primary tumor, progressive genetic and epigenetic changes take place in tumor cells (upper panel). In parallel, the stroma associated with the tumor cells also becomes progressively modified, for example through extracellular matrix remodeling and the activation and recruitment of cells such as carcinoma-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs) and other cells of the immune system. For the sake of clarity, only some of these changes have been depicted. Interactions between the tumor stroma and tumor cells modify the stemness properties of the tumors cells, for example through epithelial–mesenchymal and mesenchymal–epithelial-like transitions (EMT and MET), which regulates the cancer stem cell (CSC) subpopulation. Dissemination from the primary tumor begins early. Upon entering the secondary site, disseminated tumor cells (DTCs) have a number of possible fates, some of which are depicted in the left hand side box, bottom panel. These initial fates are determined by the underlying genetic and epigenetic status of the DTC, and through interactions with the microenvironment at the secondary site. EMT- and MET-like transitions, further genetic progression in the tumor cells, and stromal progression in the microenvironment can subsequently alter DTC fate and lead to the growth of overt metastases. Continuing mutual interactions between tumor cells and stromal cells at the secondary site stimulate stromal progression, as in the primary tumor. Furthermore, as the primary tumor grows, increasing amounts of metastatic niche-inducing factors accumulate systemically, resulting in induction of stromal progression at the secondary site, and metastatic niche formation. Metastatic niche formation can occur prior to settling of DTCs at the secondary site, as in the case of pre-metastatic niches that prime the microenvironment to support outgrowth of incoming DTCs. Self-seeding of the primary tumor or metastasis, or cross-seeding between the primary tumor and its metastasis may contribute to the overall make-up of the tumor cell population at the primary and secondary sites.

stromal compartment, the genetic and epigenetic changes in tumor cells are insufficient to support tumor growth and survival. Tumor progression is therefore built on a foundation of genetic and epigenetic changes in tumor cells, but is also absolutely dependent on stromal progression in parallel (Fig. 1). An important result of the interplay between tumor cells and the stroma is the generation of CSCs that drive tumor growth, whose properties are determined by their underlying genetic makeup, but also by the microenvironment, in a process that involves dynamic EMT and MET transitions that may only be partial. These transitions also contribute to tumor cell survival, and regulate dormancy, invasiveness and therapy resistance, and can occur in both CSC and non-CSC populations. This aspect of the stromal progression model has parallels with the dynamic heterogeneity model proposed decades ago [29].

Cancer cell dissemination begins early, for example after escape from oncogene-induced senescence [43], and continues throughout tumor growth and progression. CTCs leaving the tumor no longer have contact with the supportive stromal microenvironment they are accustomed to, and the genetic and epigenetic changes they carry are usually insufficient to support their survival or growth as a fulminant metastasis. An appropriate stromal compartment therefore has to be re-established at secondary sites if DTCs are to survive and grow out as metastases. DTCs that do not end up in an appropriate microenvironment (or which cannot initiate one) either die or remain dormant, probably eventually

regressing. If the microenvironment supports the survival of the DTCs, or is modified to support their survival, then the DTCs can continue to acquire genetic mutations and aberrations at secondary sites, and progress genetically in parallel to tumors cells in the primary tumor, as foreseen in the parallel progression model. However, concurrent stromal progression also accompanies these genetic changes in the tumor cells at the secondary site, similar to the case in the primary tumor (Fig. 1).

Stromal progression also takes place at secondary sites to form microenvironments that support outgrowth of metastases. Such microenvironments may be initiated and developed in a number of conceivable ways: (i) DTCs may settle in pre-existing microenvironments that provide stromal components they need. These may be normal stem cell niches, for example, or pre-metastatic niches induced by the primary tumor. (ii) Factors produced by the DTCs themselves may act on the surrounding stroma and initiate or contribute to the stromal progression that ultimately supports secondary tumor growth. Thus genetic changes in tumor cells can promote stromal progression. (iii) Tumor–stroma interactions in the primary tumor produce increasing quantities of soluble factors as the tumors grow, such as growth factors, cytokines and chemokines. These begin to accumulate systemically and ultimately induce the formation of metastatic niches as described above, either pre-metastatically or after the dissemination of DTCs. Hence the size of the primary tumor correlates with the incidence of metastasis, as size is proportionate to the amount of factors produced. (iv) Once a primary tumor has been removed, parallel genetic progression in the DTCs and/or associated stromal progression may eventually lead to outgrowth of metastases. In addition, other pathological events such as tissue trauma or chronic inflammation may release sufficient systemic levels of growth factors and cytokines that induce metastatic niche formation such that metastatic niche formation is kick-started and/or stromal progression is supported. Removal of the primary tumor may also remove circulating factors that were repressing distant metastatic outgrowth, such as angiogenesis inhibitors or matrix remodeling enzymes.

In the stromal progression model, formation of (pre)metastatic niches can constitute an important component of the stromal remodeling required at secondary sites for the outgrowth of metastases (Fig. 1). As in the primary tumor, the interaction of tumor cells with the stromal microenvironment at these sites plays a key role in regulating metastable EMT–MET-like transitions that determine stemness properties, control dormancy, provide survival functions and modulate resistance to therapy. Thus EMT can endow CSCs in the primary tumor with migratory properties that can be reversed at secondary sites through MET in response to a new microenvironment, as has been suggested [19]. In the absence of MET, these cells may remain dormant due to the quiescence-promoting effects of EMT. Similarly, non-CSC DTCs that survive may eventually acquire stemness properties, for example through epigenetic changes in response to EMT induced when an appropriate stromal environment develops, and/or through genetic changes. Hence the properties of the tumor cells, the nature of the surrounding stroma, the interaction between the two compartments, and the continuing interdependent progressive evolution of the tumor cells and the tumor stroma act together to determine the stemness properties required for the outgrowth of metastases, regulate the re-activation of dormant cells and determine sensitivity to therapy. Like primary tumors, metastases may disseminate cells, and cross-seeding between primary tumor and their metastases may contribute to the similarities between them that are observed histologically and in transcriptomic studies.

The stromal progression model suggests that the sparse existence of appropriate endogenous stromal microenvironments that are able to support tumor growth contributes to the low efficiency of metastasis formation in experimental metastasis assays. This may also be a reason why large numbers of cells are required to get an efficient "take rate" in experimental animals, and why providing constituents of a supportive stroma, for example in the form of Matrigel, increases take rate. The model also provides an explanation for why continuous passaging of tumor cells in experimental animals and selection for growth in particular organs would give rise to tumor cells that metastasize efficiently to the organ in question. Here, tumor cells are selected that have the ability to interact with particular stromal microenvironments of the organ concerned, to induce stromal progression in those microenvironments, and/or to undergo genetic or epigenetic changes in response to the endogenous or induced microenvironment.

6. Concepts and clinical perspectives

While the stromal progression model incorporates many theories, observations and experimental findings, several open questions remain. Major issues include the timing of dissemination of the metastatic seed, the degree to which the regulation of stemness properties overlaps with the pathways that control EMT, dormancy and therapy resistance, key stages in stromal progression, and the mutual interdependence between genetic changes in cancer cells and changes in the associated stroma. Understanding how metastasis works is of more than just academic interest, as an accurate conceptual grasp of the process is fundamental to effective therapy. For example, if the tumor cells that seed metastases disseminate late, a window of opportunity opens to remove the primary tumor before metastatic deposits have taken root. If on the other hand, early dissemination and parallel progression is the overriding mode of metastatic seeding, then at the time of cancer diagnosis, DTCs with the potential to develop into metastases will already be present, and therefore the therapeutic strategy will need to be different. Another implication of parallel progression is that the choice of targeted therapies to treat metastases should be based on molecular and biological features observed in metastases rather than in primary tumors [22].

The dormancy of DTCs over long periods of time and their relative stability, together with relapse occurring many years after diagnosis, surgery and initial treatment demands that more effort is placed on understanding the regulation of dormancy. This may provide a novel opportunity to prevent metastatic outgrowth and keep disseminated cancer as a dormant, chronic but manageable disease. Key issues are to understand how quiescent, disseminated cancer cells interact with the microenvironment, and to define the critical cues that awake cancer cells form dormancy and allow them to progress to full metastasis.

Understanding the nature of the tumor cells that initiate metastases could be key to successful therapy. If metastases are seeded by particular CSC subpopulations, then targeting them would be expected to effectively suppress metastasis formation. The expression on CSCs of specific members of the family of CXC chemokines receptors has recently received interest in this regard. Chemokines serve as chemoattractants for cells endowed with CXC receptors such as CXCR4 and CXCR1 that have been found to earmark migratory subpopulations of CSCs in pancreatic and breast cancer, respectively [47,168]. Selective blockade of CXCR1 targets breast CSCs in human xenografts slow down primary tumor growth and reduce metastasis formation [169]. Clinical trials with pharmacological inhibitors and monoclonal antibodies directed against specific CXCRs will assess their capacity to block CSCs dissemination and prevent metastasis formation in cancer patients. These and similar studies may provide novel therapeutic strategies to selectively target cancer CSCs after dissemination throughout the body of the cancer patient and prevent them from forming distant metastases.

As pointed out above, the genetic disparity between primary tumors and their metastases suggests that analysis of the primary tumor may not be the best way to determine appropriate treatment of metastatic disease. CTCs represent tumor cells that have left the primary tumor and are also likely to be derived from metastases, so there is growing interest in monitoring CTC as cellular surrogates of metastatic dissemination [170]. DTCs are much less accessible than CTCs, and can be less informative [171].

While CTCs can be detected in the blood of patients with many types of solid cancer, they are best characterized in breast cancer patients and most of our knowledge on CTCs is derived from breast cancer [172,173]. Strong evidence indicates that the number of CTC before treatment is an independent predictor of progression-free survival and overall survival in patients with metastatic breast [174] or prostate [175] cancers. Subsequently it has been shown that detection of even rare CTCs is associated with an increased risk of metastatic progression and reduced survival in newly diagnosed breast cancers [176,177]. A clinical challenge here is to define whether CTC can be developed as reliable surrogate marker of relapse and progression to metastasis for individual patients with primary breast cancer undergoing adjuvant treatments. Several clinical trials are currently addressing this question [173]. Another equally challenging and relevant issue relates to the potential clinical use of CTC as biomarker to predict response to therapy in metastatic cancers. Initial evidence indicates that this might be the case in breast cancer, as persisting elevated counts of CTC during therapy predicts shorter progression-free survival and precedes radiological signs of progression [178]. Additional studies are in progress [173].

While cumulating evidence indicates that CTC counts have prognostic and predictive clinical significance, many important questions on the biology of CTCs remain unanswered. For example, what is the best method to detect CTCs? CTCs are rare in the peripheral blood (ranging from one to hundreds of cells per ml) and reliable detection/isolation is still challenging [179]. Available methods are mostly based on immunomagnetic isolation using antibodies directed against the epithelial cell surface molecule EpCAM (such as the commercially available and FDA-approved system CellSearch®), followed by immunocytochemistry staining for epithelial markers (*e.g.* CK 8, 18, 19) [173]. As some CTCs undergo EMT, this approach may miss an important CTC subpopulation. Similar arguments also apply to the analysis of DTCs. Thus, novel enrichment strategies including EMT markers need to be developed.

A second crucial question is whether all detected CTCs are potentially able to colonize distant organs and form metastases. In other words, is the number of CTCs sufficient to predict metastasis, or should additional biological and molecular parameters also be considered? Determining viability, proliferation and expression of EMT or stem cell markers may already improve the prognostic/predictive power of CTC, but the ultimate test would be to directly evaluate the metastatic capacity of individual CTCs in laboratory assays.

A third outstanding issue is whether CTCs represent a more appropriate cell population to define therapeutic strategies, compared to cancer cells in the primary tumor, which are currently used for this purpose. The relevance of this point is exemplified by the detection of HER-2-positive CTCs in patients with HER-2-negative primary breast cancer and, conversely, HER-2-negative CTCs in patients with HER-2-positive tumors [180–182]. CTCs may also be used, for example, to validate the activity of targeted anticancer drugs, for instance by monitoring the phosphorylation state of kinases targeted by the drugs or their downstream effectors [183].

In summary, clinical and basic research into the underlying mechanism of metastasis has in the last few years unearthed many new facets of the process that results in the formation of secondary cancers. While we are still some way from a complete understanding of the metastatic process, it is clear than many of the contemporary models and theories that have arisen as a result of these new findings are starting to converge. The stromal progression model we suggest here integrates many of these ideas. The next few years will see exciting further progress that will provide us with an increasingly accurate concept of how metastasis works, which in turn will allow rational and effective therapies for metastatic disease to be developed.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

All authors gratefully acknowledge funding from the European Union under the auspices of the FP7 collaborative project TuMIC, Contract No. HEALTH-F2-2008-201662.

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