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Epithelial-Mesenchymal Transition: A Hallmark in Metastasis Formation Linking Circulating Tumor Cells and Cancer Stem Cells

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Abstract
The occurrence of either regional or distant metastases is an indicator of poor prognosis for cancer patients. The mechanism of their formation has not yet been fully uncovered, which limits the possibility of developing new therapeutic strategies. Nevertheless, the discovery of circulating tumor cells (CTCs), which are responsible for tumor dissemination, and cancer stem cells (CSCs), required for tumor growth maintenance, shed light on the metastatic cascade. It seems that CTCs and CSCs are not necessarily separate populations of cancer cells, as CTCs generated in the process of epithelial-mesenchymal transition (EMT) can bear features characteristic of CSCs. This article describes the mechanisms of CTC and CSC formation and characterizes their molecular hallmarks. Moreover, we present different types of EMT occurring in physiological and pathological conditions, and we demonstrate its crucial role in providing CTCs with a CSC phenotype. The article delineates molecular changes acquired by cancer cells undergoing EMT that facilitate metastasis formation. Deeper understanding of those processes is of fundamental importance for the development of new strategies of early cancer detection and effective cancer treatment approaches that will be translated into clinical practice.

Introduction

Distant metastasis development is the main cause of death in cancer. Metastatic lesions are hardly manageable in clinical practice as they are often too widespread or too large to be removed by surgery and frequently exhibit increased resistance to chemotherapy. Thus the understanding of the metastasis process is of key significance for treatment improvement and decreasing the death rate of cancer patients.

In order to progress, tumors of epithelial origin need to acquire features which enable them to: (1) loosen cell-cell contact, breach the basal membrane and dissociate from the tumor mass, (2) invade neighboring tissue, intravasate into blood or lymph vessels and (3) extravasate

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from vessels in distinct organs to create secondary tumor(s) [1]. Epithelial tumor cells gain invasiveness and migratory abilities in the process of epithelial-mesenchymal transition (EMT), which is essential for successful metastatic spread [2, 3]. The process of EMT is very likely to be responsible for drug efficiency decrease and thus anticancer therapy failure. Moreover, it appears that stem-like cells can arise as a result of EMT [4, 5]. Circulating tumor cells (CTCs), isolated from the blood of breast cancer patients, may be linked to both cancer stem cells (CSCs) and EMT processes as they can possess features of CSCs as well as phenotypic changes characteristic of EMT [6–9]. Altogether, these cells are predestined to be an active source of metastases due to their potential stem cell features and EMT traits which allow them to disseminate effectively.

A considerable effort has been dedicated to elucidating the molecular background of tumor invasion and metastasis, and an enormous amount of data on the subject has been generated. Here we review the emerging picture of EMT as the hallmark in metastasis formation, linking both circulating tumor cells and the formation of cells with cancer stem cell phenotypes. We delineate the clinical significance of CTCs as such and their molecular characteristics as predictive and prognostic tools in cancer management.

EMT in Physiology and Pathophysiology

EMT is a multi-step process involving molecular and cellular changes in epithelial cells. Restrained and immobile epithelial cells gain a mesenchymal phenotype, characterized by an enhanced motility and ability to degrade ECM [10–15]. This leads to the weakening of cell-cell adhesion due to the downregulation of epithelial proteins, mainly E-cadherin but also claudins, occludins and cytokeratins [2, 15–17]. Cells that have undergone EMT show changes in apicobasal polarity which leads to a spindle-shaped morphology [18, 19]. The characteristic features of a mesenchymal phenotype are high expression of N-cadherin as well as fibronectin, vimentin, tenascin C, collagen VI-α and laminin-β1 [13, 16, 17, 20, 21]. These changes observed in the EMT process are governed by transcription factors such as TWIST1, SNAIL, SLUG, SIP1 [14, 22]. To date a number of different EMT inducers such as Wnt, Hedgehog, EGF and TGF-β have been unveiled [23, 24] and molecular pathways have been delineated. For example, the binding of TGF-β to proteins expressed on the surface of breast cancer cells activates the expression of SNAIL and SLUG which suppress E-cadherin expression [25].

EMT plays a critical role in a number of physiological and pathophysiological conditions. Depending on the mechanisms, different classes of EMT have been proposed: embryonic and developmental EMT referred to as type I, fibrotic and wound-healing EMT referred to as type II and cancer progression EMT known as type III [11–14, 26, 27].

EMT type I (classic EMT) besides embryonic development also occurs during postnatal growth. Furthermore, it plays a role in maintaining epithelial homeostasis. The steps of EMT during this process are specific and well defined. Normal epithelial cells are cuboidal and remain in contact with each other through adherent and tight junctions. They are also attached to the basal membrane by integrins. Adherent junctions are composed mainly of E-cadherin, catenins and actin, while claudin and occludins are more characteristic of tight junctions. EMT inducers downregulate the expression of the components of both tight and adherent junctions, which results in loosening of the apicobasal polarity of the cells and disassembling of the basal membrane [28, 29]. This process is accompanied by changes in the cytoskeleton, such as the replacement of peripheral actin elements by stress fibers, which are crucial for cells to liberate from the epithelium and acquire migratory properties [22, 30]. All the aforementioned changes, together with simultaneous protease activity, lead to cell delamination and invasion [31]. Primary mesenchymal cells generated in this way have the potential to subsequently go through the process of mesenchymal-epithelial transition (MET), the reverse of EMT, and thus generate secondary epithelia [27]. During embryonic development, cells may undergo EMT a number of times; hence, almost all organs in adults arise as a result of one or several rounds of EMT followed by MET.

The ability of cells to undergo the process of EMT and subsequently reverse is called epithelial plasticity. A unique example of this phenomenon is the postnatal development of the mammary gland. The developmental cycle of the breast is long-lasting, since the mammary gland achieves its full dimensions after the first lactation during mammary gland involution. During this complex organ evolution, epithelial cells need to go through many rounds of proliferation, invasion and cell death [32, 33].

EMT type II (fibrotic EMT) is triggered by injury and mainly generates fibroblasts in order to reconstruct wounded tissues [3, 12, 34, 35]. In physiological conditions fibroblasts and immune cells release inflammatory factors (e.g. different cytokines) and extracellular matrix

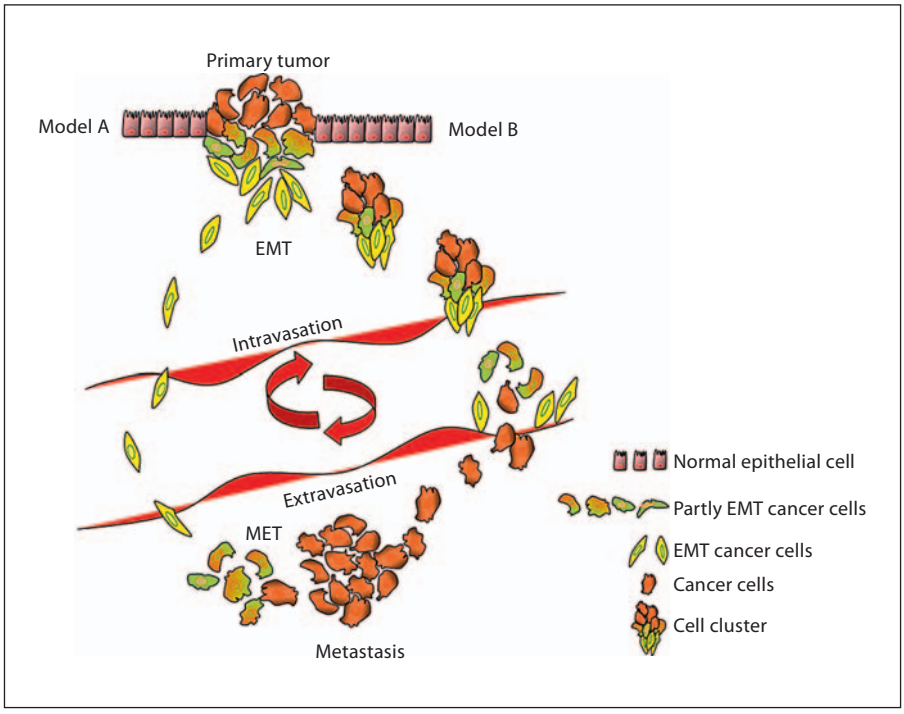

Fig. 1. The role of EMT in cancer invasion. Two models have been proposed for EMT involvement in cancer metastasis. After stimulation, carcinoma cells activate a program of EMT and phenotypical changes occur, leading to the formation of either a pure population of highly invasive mesenchymal-like cells or sets of cell clusters with 'hybrid' phenotypes. Model A: after undergoing full EMT, mesenchymal-like cancer cells disseminate from the tumor mass and reach the circulatory system. Once they extravasate in a distant organ, they undergo MET and create metastases [12, 50]. Model B: cancer cells disseminate and migrate as clusters of cells with different phenotypes; cancer cells that pass through full EMT lead the partial EMT and non-EMT cells. All cell types enter the circulation, but only non-EMT cancer cells are able to extravasate and create metastases [55].

Color version available online

proteins which stimulate cells to undergo EMT. Once the inflammation subsides the process is terminated. Under pathological conditions of persistent inflammation, continuous EMT of normal epithelial cells can lead to fibrosis and organ (lung, liver, kidney) damage [3, 12, 34–37].

Similarly to fibrosis, the oncogenic process can disturb homeostasis in cells and induce EMT type III. The oncogenic EMT enables epithelial cells to acquire motile and invasive phenotype characteristics for mesenchymal cells, which is essential in metastatic cascade [2]. Many EMT features typical for development are present in oncogenic EMT, however, they are less ordered and coordinated. Although it seemed to be predictable that the same mechanism could be responsible for the delamination of cells, both in the development and metastatic spread of epithelial cancers, the contribution of the EMT process to tumor progression has only recently been commonly accepted [38–40]. This was caused by the transient character of EMT in tumors and technical difficulties in identifying migrating cancer cells on pathological specimens [4, 41–43]. EMT occurs in restricted places within a tumor (such as a leading edge [44]) and the micrometer-thick slices allow observation of only a limited area of a tumor cross section. However, the altered shape of tumor cells and the process of intravasation have been shown in vivo in animal models using intravital microscopy, which allows real-time monitoring of fluorescently labeled tumor cells, and adjustment of the depth of focus and contrast [45]. Moreover, the lack of direct clinical evidence for EMT in cancer was boosted by the observation that secondary tumor sites histopathologically resemble cells of the primary tumor from which they originated. This can be explained by the occurrence of the MET process once tumor cells have extravasated into distant organs [27]. Disseminated cancer cells need to regain their epithelial phenotype in order to initiate the growth of a solid tumor at the secondary site. The studies of Chao et al. [46] demonstrated that the secondary organ microenvironment can induce the process of MET in the mesenchymal-like MDA-MB-231 breast cancer cell line and in primary explants by reexpression of E-cadherin. A few other studies have described the phenomenon of a switch between EMT and MET in bladder, colorectal and ovarian cancer [39, 47, 48] (fig. 1, model A). It should also be mentioned that changes between epithelial and mesenchymal phenotype in carcinoma cells may lead to the arising of hybrid phenotypes which 'accumulate' features of both cell types [18, 49, 50]. Cells do not need to undergo a full transition to a mesenchymal phenotype or even to show any changes typical of EMT. In fact, they can stay morphologically well differentiated and take part in the metastasis process. This process is known as 'collective'

or 'cohort' migration in which cells do not disseminate and invade as individuals but as multicellular clusters. It is possible that in the cluster of migrating cells many intermediates between epithelial and mesenchymal phenotypes coexist. The invasive front of the tumor consists of mobile invasive cells resulting from EMT, followed by unchanged tumor cells with epithelial characteristics [50–52]. This type of cell migration occurs both in breast cancer and during regular development [53, 54]. An interesting model of collective migration in cancer was described by Tsuji et al. [55] where only cooperation between EMT and non-EMT cancer cells enabled a successful process of metastasis. EMT cells with an invasive phenotype, responsible for matrix degradation and penetration of local tissue and vessels, lead non-EMT cells and enable their intravasation. Non-EMT cells endowed with adhesive properties are able to attach to the vessel wall and extravasate, successfully creating metastasis (fig. 1, model B). During breast cancer development, both the EMT process and partial EMT can be observed. EMT, for example, is representative of infiltrating lobular carcinoma. Partial EMT manifests itself as cell cohorts and partially dedifferentiated tubules found in invasive ductal carcinoma [50]. The existence of more than one cellular mechanism for tissue invasion is obvious both in branching morphogenesis and in epithelial cancers; however, a lot of effort must be put into understanding the dependence of these processes.

Cancer Stem Cells and Metastasis

In the last decade the understanding of the metastasis process has changed considerably. A stochastic model of tumor development and maintenance was challenged by 'cancer stem cell hypothesis' [56–58]. The stochastic model assumes that all cells in cancer are equally malignant and every single cell has the potential of reconstituting a primary tumor under favorable circumstances, although the probability of this event is low [59, 60]. According to this concept, heterogeneity within the population of tumor cells develops during tumor progression due to the effect of genomic instability and the accumulation of mutations. Then, in the process of clonal selection, the tumor is enriched for cells endowed with metastatic properties, which can disseminate and form secondary tumors [61, 62]. 'Cancer stem cell hypothesis' implies the preexistence of functional heterogeneity within tumor cells with a discrete subpopulation of CSCs, able to initiate and maintain tumor growth and bulk nontumorigenic cells [57, 58, 63–66]. Evidence for the existence of CSCs was first reported by Bonnet and Dick [58] in acute myelogenous leukemia, and then shown for breast cancer [57] and other malignancies [64–67]. CSCs are so termed through their analogy with normal stem cells. Similarly, CSCs possess the ability to self-renew in vivo and differentiate. They can give rise to a phenotypically diverse progeny composed of both tumorigenic cells with indefinite proliferation potential and nontumorigenic cells with limited proliferation potential. This way they recreate the whole repertoire of cell subpopulations observed in the original tumor. However, in contrast to normal stem cells, they do not need to exhibit multilineage differentiation ability [58, 68, 69]. In spite of their name, CSCs do not necessarily arise from normal tissue stem cells. They can also originate from more differentiated progenitor cells which underwent transformation [70, 71]. Alternatively, CSCs may arise through an EMT process from transformed epithelial cells and achieve migratory and tumor-spreading properties [5, 72, 73]. In an experimental system, the induction of EMT in immortalized, nontumorigenic human mammary epithelial cells resulted in acquisition of the $CD44^+/CD24^{-/low}$ phenotype, characteristic of breast cancer stem cells [4]. CD44 is a cell-adhesion molecule involved in binding cells to hyaluronic acid, whereas CD24 is a negative regulator of the chemokine receptor CXCR4, a molecule involved in breast cancer metastasis [74]. It must be kept in mind that the heterogeneity of tumorigenic ability existing between different types of breast tumors must not necessarily be related only to the $CD44^+/CD24^{-/low}$ phenotype. It has been shown that the $CD44^+/CD24^{-/low}$ subpopulation of cells is present in most (but not all) basal-like tumors, especially in BRCA1 hereditary breast cancer. However, this subpopulation of cells is present in only a few HER2-positive tumors [75]. Ever since Al-Hajj et al. [57] defined the CSC subpopulation in breast cancer on the basis of CD44 and CD24 expression, their status has been examined in other tumors. Cells with the $CD44^+/CD24^{-/low}$ phenotype were postulated to be stem-like cells responsible for tumor initiation in non-small cell lung cancer and prostate cancer [76, 87]. Interestingly, in pancreatic cancer, a subpopulation with a high coexpression of CD24 ($CD44^+/CD24^+/ESA^+$) was identified as being endowed with tumorigenic activity in NOD/SCID mice [78]. Similarly, in gastric cancer $CD44^+/CD24^+$ cells were recognized as cells with stemness features [79]. CD44 and CD24 are also listed among colon CSC markers, although combined with CD166 and CD133, respectively [80, 81]. Expression of CD24, CD44 and CD133 is correlated with

the invasiveness and differentiation of colorectal carcinoma, but not with patient outcome [82]. The list of stemness markers is still growing, both cancer type-specific and universal. Mostly, markers have been identified through the exploration of cell surface proteins and since many of them are important for cellular adherence (CD24, CD133, CD166) they are probably involved in forming new tumors [83]. However, focusing on markers involved only in the cell attachment can be a source of false conclusions since the enhanced ability of these cells to grow in new environments does not necessarily need to be a hallmark of stemness itself. It is also important to identify functional markers for CSCs, to which, for example, ALDH1 belongs (present in breast, colon, pancreatic carcinoma [84–86]) or *Wnt* [61, 90]. ALDH1 is a marker for both normal and malignant stem cells and it is thought to have a role in the early differentiation of stem cells [84, 88]. Within breast cancer cells with a $CD44^+CD24^{-/low}$ phenotype, a subpopulation of ALDH1-positive cells endowed with a prominent tumor-initiating ability was found. Moreover, expression of ALDH1 alone in breast cancer samples correlated with poor clinical outcome [88]. Other proteins proposed as stemness markers in breast cancer include the following: CD133 [89], OCT-4 [90] and NANOG-1 [91]. OCT-4 is a transcription factor taking part in the self-renewal of undifferentiated embryonic stem cells and its expression can reprogram unipotent stem cells to pluripotent cells [92]. High expression of OCT-4 was observed in $CD44^+/CD24^-$ cancer cells isolated from primary breast cancer tumors [93] and in ALDH1-expressing cells of the 4T1 murine breast cancer cell line [94]. OCT-4 can also act together with another transcription factor involved in the pluripotent state of stem cells – NANOG-1 [95, 96] – and their expression is elevated in mammospheres, which are formed by cells enriched for metastatic potential [94, 97]. CD133 function is not yet well understood, however, it might be involved in cell differentiation and epithelial-mesenchymal interaction [98]. CD133-positive breast cancer cells frequently express other stem cell markers – *NOTCH1*, *ALDH1*, *SOX1, CD44* – and are highly tumorigenic in animal models [99].

Features of CSCs such as motility, invasion, survival in circulation, dormancy and ability to interact with micromilieu at a secondary tumor location imply that these cells might be responsible for the development of overt metastasis [56, 100–102]. Such a mechanism, explaining metastatic spread through the existence of mammary stem and progenitor cells already at the beginning of tumor transformation, is supported by experiments employing gene expression profiling. Liu et al. [103] developed a gene signature which consists of 186 genes differentially expressed in normal breast epithelium and in breast $CD44^+/CD24^{-/low}$ cells. This signature was significantly correlated with metastasis-free and overall survival. Another study employing microarray analysis of $CD44^+CD24^{-/low}$ cells versus $CD44^-/CD24^+$ cells isolated from breast cancer tumors confirmed that these two subpopulations of cells are genetically distinct from each other, and the gene expression profile of the breast cancer stem cell fraction ($CD44^+/CD24^{-/low}$) resembles a profile characteristic of stem cells [104]. Analogically to normal breast stem cells, this subpopulation of breast cancer cells was able to form mammospheres in vitro [4, 105].

CTCs and Metastasis

CTCs are cells which manage to separate from the tumor mass and enter the bloodstream. They are defined as tumor cells originating from either primary sites or metastases and circulating freely in the peripheral blood. They have been detected in a majority of epithelial cancers, including those from breast, prostate, ovary, lung and colon, but are extremely rare in healthy people [106]. CTCs may constitute seeds for the subsequent growth of metastasis in distant organs according to Paget's 'seed and soil hypothesis' [107]. However, CTCs may also be capable of self-seeding back to the original organs, which infers increased aggressiveness of the existing tumor [108], or they can settle in other organs such as bone marrow, a point at which they are termed disseminated tumor cells (DTCs) [109] and can serve as a reservoir of tumor cells responsible for future recurrence [110].

CTCs represent a heterogenous population of tumor cells with the potential of forming various metastases. From experimental models it is estimated that about 1 million cells per 1 g of tumor tissue can spread daily into the bloodstream [111]. In mouse models where cells were injected intraportally, only 1 CTC in 40 was able to establish metastatic foci, and 1 in 100 micrometastases could form a tumor at day 13 after injection [112]. This metastasis inefficiency is mainly the effect of anoikis and explains the low survival rates of CTCs in vessels after leaving the tumor mass [112–114]. Moreover, only some solitary cells which extravasate in distant organs are able to proliferate, and finally, not every established micrometastasis can overcome the step of new vessel formation and develop into macrometastasis [112].

The first document describing CTCs in the peripheral blood of cancer patients dates from 1989 [115] and since that moment the interest in the clinical significance of CTCs has been increasing. CTCs are called 'liquid biopsy' since they are a potential alternative to invasive biopsies as a source of tumor material for the detection, characterization and monitoring of nonhematologic cancers. The clinical applications of CTCs are strictly dependent on the development of reliable techniques for CTCs detection.

Despite huge efforts and numerous studies, CTCs detection is still technically challenging, mainly due to their paucity and biological heterogeneity [116]. Currently used approaches for CTCs isolation and detection are based on the properties which distinguish CTCs from abundant blood cells. This includes the presence of specific proteins (CellSearch, CTC-chip, RARE, MagSweeper) and gene transcripts (AdnaTest), size (ISET technology), density (Oncoquick), electric charge, secretion of specific proteins (EPISPOT) or invasive properties [117]. There are many issues concerning reliable CTC detection which are still a matter of debate. They concern the accuracy, sensitivity and specificity of the techniques, the optimal cutoff for CTC enumeration, optimal markers for CTC identification or ability to determine the cell condition (viable or apoptotic, dividing or nondividing). Unfortunately, there is a great interlaboratory variability regarding the techniques used, and no tumor marker identified so far is specific enough to detect rare CTCs. The most widely used approach relies on the epithelial molecule – EpCAM – the expression of which is present in 60–100% of breast cancers [118, 119]. However, the study of Sieuwerts et al. [120] showed that EpCAM-based CTC detection does not recognize breast cancer cells belonging to a normal-like subtype, which is characterized by aggressive behavior. This deficiency of assay sensitivity can be overcome by using the additional surface marker CD146, which is frequently expressed on cells lacking EpCAM [121]. Additional drawbacks of the epithelial marker-based isolation and detection methods were revealed when it was discovered that the EMT, which occurs in CTCs, causes downregulation of epithelial markers and renders CTCs undetectable. Therefore, experiments aimed at the analysis of the EMT process itself, or the EMT-induced stemness of CTCs, should not rely solely on a single marker but on a few markers [122, 123], and possibly also include mesenchymal markers induced by EMT [116]. There is an urgent need for optimization and clinical validation of the developed techniques of CTC detection and isolation. Progress in this matter will largely depend on better characterization of CTC phenotypes and deeper understanding of the mechanisms involved in their generation and survival.

Currently, it is evidently clear that cells detach from tumors well before a metastasis is clinically visible [124, 125]. Therefore, CTCs enable tracking of one of the first steps in a metastatic cascade and provide great potential for prognosis and monitoring of treatment response in many cancers. The presensce of CTCs was demonstrated to be an independent adverse prognostic factor in metastatic [126–128] and early breast cancer [129], metastatic colorectal cancer [130], castration-resistant prostate cancer [131, 132] or resectable non-small cell lung cancer [133, 134].

Since CTCs can be obtained repeatedly in a noninvasive manner, they may be used to choose the optimal therapy, predicting the response to it and monitoring its efficacy. The number of CTCs in patients with breast, lung, prostate and other cancers was decreased after the initiation of effective chemotherapy, hormonal therapy or targeted therapy [125, 127, 128, 135, 136]. Treatment-induced changes in CTCs numbers measured at different time points were also proven to be a reliable surrogate marker of response to treatment [137–139].

Not only enumeration of CTCs, but also determination of their molecular profiles provides information which is important from a clinical point of view. It was found that CTCs may be genetically different from the primary tumor they derive from, and that the differences might influence patient response to therapies, which are currently prescribed on the basis of the primary tumor characteristics. For example, HER2-positive CTCs were reported in breast cancer patients who had HER2-negative primary tumors, and vice versa, HER2-negative CTCs were observed in patients with HER2-positive tumors [140, 141]. Analogical dissimilarities between CTCs and primary tumors have been demonstrated for epidermal growth factor receptor (EGFR) [142], estrogen receptor alpha, and progesterone receptor [143]. These differences might be explained by the strong selection of a specific cell population during dissemination or by the methodological limitations of a biomarker (such as HER2) determination [116]. In addition, CTCs may also get to the blood circulation from secondary sites [108], and may be similar in genotype and phenotype to the cells from metastatic sites instead of the primary origin.

Moreover, the characterization of CTCs may allow examination of the molecular evolution of tumor cells during the course of treatment, which may be particularly important in monitoring of the development drug resistance [136]. Recently, real-time CTCs genotyping during

treatment with EGFR-targeted therapies against non-small cell lung cancer [136] and breast cancer [144] has been reported. Thus, it seems that treatment decisions may be based on the molecular profile of CTCs. The ongoing analyses of the results of clinical trials, such as the Southwest Oncology Group trial SWOG S0500 (www.clinicaltrials.gov, identifier: NCT00382018) on metastatic breast cancer patients, the German Breast Group GEPARQuattro trial (www.germanbreastgroup.de/en/trials/neoadjuvant/geparquattro.html) and the SUCCESS trial on nonmetastatic breast cancer patients (www.success-studie.de), should provide more comprehensive knowledge of the CTC-based tailoring of treatment.

Recent studies have revealed that CTCs may be linked to both cancer stem cells and the EMT process [13, 145], which adds additional value to the clinical utility of CTCs. The expression of EMT-related proteins, such as vimentin or TWIST1, has been found in CTCs obtained from breast cancer patients [146, 147]. CTCs with a hybrid (epithelial/mesenchymal) phenotype have also been found in patients with metastatic non-small cell lung cancer [148] and prostate cancer [9]. Interestingly, mesenchymal markers on CTCs occur more frequently in metastatic breast cancer than in breast cancer at an early stage [146], and allowed more accurate prediction of worse prognosis than the expression of epithelial markers alone [147]. In patients with primary breast cancer the overexpression of EMT-inducing transcription factors (TWIST1, SNAIL1, SLUG, ZEB1, and FOXC2) was more frequently detected in those who received neoadjuvant therapies than in those who did not, which suggests that neoadajuvant therapy is unable to eliminate CTCs undergoing EMT [149]. Moreover, the overexpression of EMT markers on CTCs was often accompanied by the presence of the stem cell markers ALDH1 in breast cancer at all stages of the disease [6–8] and CD133 in castration-resistant prostate cancer [9]. The existence of a subpopulation of CTCs with a stem cell-like $CD44^+CD24^-$ phenotype and ALDH1 expression has also been described in metastatic breast cancer [150]. In addition, CTCs from patients with primary or metastatic breast cancer have been shown to express receptors and activated signaling kinases of the EGFR/HER2/PI3K/Akt pathway [142], which is one of the major pathways involved in the regulation of mammary stem/progenitor cells, promoting the proliferation and inhibition of apoptosis [151]. It has recently been demonstrated that, similarly to mammary stem cells [152], CTCs found in primary breast cancer patients are mostly triple-negative – estrogen receptor-negative, progesterone receptor-negative and HER2-negative [153].

The data concerning the occurrence of EMT in DTCs are scarce and not conclusive. Cell lines derived from DTCs of breast cancer patients show low expression of CK8, CK18 and CK19 cytokeratins, and increased levels of vimentin, which resembles characteristic features of EMT [154, 155]. Moreover, elevated TWIST1 expression was found in EpCAM-enriched bone marrow samples [156], which suggests the occurrence of partial EMT. Additionally, an increased tumor-initiating ability of DTCs, recognized as the stem cell phenotype ($CD44^+CD24^-$), was detected in both the DTC-derived cell lines [156] and the CK19-positive DTCs isolated from the bone marrow of early-stage breast cancer patients [157].

To sum up, the expression of stemness markers and EMT markers in CTCs might provide them with strong metastasis-initiating properties, and render them resistant to conventional anticancer therapies. The described findings also demonstrate that current CTC detection methods may lead to underestimation of the significance of the most important, EMT-positive, subpopulation of CTCs involved in cancer dissemination. This could also explain why CTCs are currently being undetected in about 30% of metastatic breast cancer patients [141]. Thus, the fact that CTCs show stemness and EMT features is of fundamental importance for their reliable detection, which in turn can be used as a tool for tailoring effective cancer treatment.

Seed and Soil Hypothesis

Metastasis is a complex process requiring interplay between the seeded tumor cells and the microenvironment at the secondary site. Although the ability of tumor cells to migrate, either intrinsically or conferred through EMT, is of key importance for tumor progression, it is not sufficient to set up secondary tumor lesions in different anatomical compartments. A growing body of evidence indicates that the interaction between tumor cells and the local microenvironment at the secondary site leads to the development of premetastatic niches – compartment(s) of the body with a microenvironment which allows the malignant cells to develop metastases. Common sites of metastases in breast cancer include organs such as bone, the liver, lung and brain [158–160]. More than a hundred years ago, Stephen Paget noticed that the pattern of metastases produced by different neoplasms is not random. In his 'seed and soil' hypothesis, Paget claimed that certain tumor cells ('seed') have an affinity for the microenvironment of specific organs ('soil'), and only when the

'seed' and the 'soil' are compatible can metastases occur [107]. He claimed that the 'soil' properties may be of great value in understanding cancer metastasis, revealing that certain populations of cells ('seed') may be expected to play a central role in the formation of secondary tumors. Those cells may be predestinated to establish metastases in specific organs already at the initial stages of tumor development [161, 162]. Moreover, it is now known that tumor cells can produce factors that lead to the establishment of premetastatic niches [163, 164]. However, the mechanism allowing tumor cells to influence the behavior of microenvironment cells is poorly understood. Müller et al. [165] explain metastasis formation through an analogy with chemokines attracting immune cells to inflammation sites. A distinct pattern of chemokine receptor expression on tumor cells and their ligands in common sites of metastatic spread is critical for tumor progression [166]. For example, the receptor-ligand complex CXCR4-CXCL12 is well described in the context of breast cancer progression. CXCR4 is expressed on the surface of breast cancer cells [165], whereas CXCL12 is released by stromal cells in the target organs of breast cancer metastasis (bone, lung, brain, liver, lymph node) which indicates its role in targeted metastasis [165]. A high level of CXCR4 on tumor cells is correlated with poor prognosis in breast cancer patients because of its association with lymph node metastasis [167]. Moreover, it was observed that HER2, which positively correlates with metastasis and poor survival, enhanced the expression and function of CXCR4 by inhibiting its degradation [168]. The CXCR4 receptor is also considered a marker for stem cells and the subpopulation of cells expressing CXCR4 increases dramatically in secondary tumors [169]. Hence, CXCR4 expressed on stem cells allows them to follow the gradient of CXCL12 and 'seed' tumors in remote organs [170]. A recent work by Labelle et al. [171] shed a new light on the subject of the metastatic process. They demonstrated that the interactions of tumor cells with their microenvironment at the primary tumor site may also be sufficient to direct the tumor cells into the specific site of future metastasis. Platelet-derived signals (TGF-β) and direct cell-cell contact were shown to be sufficient for effective metastasis of cancer cells.

Summary

Metastasis formation is a highly inefficient process as it requires gaining several unique features simultaneously. The tumor cells not only need to acquire increased motility and invasiveness, but also have to evade the adverse conditions they encounter. At first, the tumor cells that reached the circulatory system, CTCs, are in danger of death by anoikis [172, 173], destruction by immune system cells either in the bloodstream or after extravasation [174]. Moreover, not all the disseminated cells are able to initiate and sustain proliferation that can be later seen as a macrometastasis [112]. According to the cancer stem cell hypothesis, only a fraction of continuously proliferating cells can do so. The exact mechanisms that allow cancer cells to overcome those limitations and form a metastatic lesion are not fully understood. However, it seems that one way is through cancerous EMT which allows cells to: (1) become invasive and motile, (2) become resistant to anoikis, thus enabling separate cells to exist in the form of CTCs, and (3) gain stem cell properties needed to initiate metastatic growth at a distant site. All of the mentioned features can be found in both CTCs and CSCs, which suggests that stem cell-like cells can be generated by EMT occurring in a primary tumor [174]. Although the discussion on the definition of cancer stem cells is still ongoing, as it is not clear what a cancer stem cell really is, there are markers that define the population of cells showing increased tumorigenicity. As discussed before, $CD44^{+}/CD24^{-/low}$ populations from breast cancer tumors can be referred to as possessing stem cell properties. Despite reproducible results indicating the presence of CD44 on stem cells, the protein seems to be one of many stemness markers.

Cancer stem cells may arise in different ways and during cancer development they can undergo genetic and epigenetic changes. This may result in the formation of different populations of CSCs which vary in their malignancy [175, 176]. To test this hypothesis many more markers need to be studied, which will allow more precise characterization of the tumor-initiating ability of CSCs.

Interestingly, it has been shown that in breast cancer cells the expression of markers of a tumorigenic subpopulation can change, thus changing the tumorigenic cell into a nontumorigenic cell and vice versa. Nevertheless, the proportion of CSCs to non-stem cancer cells, which is typical for the cell line, is maintained [177]. In the case of CTCs, the presence of other markers (like ALDH1, OCT-4, NANOG, CD133, CXCR4, TWIST1, or vimentin) can allow further definition of a group with a higher degree of stemness or aggressiveness.

Understanding the process of metastasis formation requires broadening the knowledge of the individual steps of metastatic cascade. Even though the mechanism of cell migration – cohort migration, observed in tumors –

seems to be reminiscent of a morphological program, single cells can also be detected in the circulation [30, 52]. These solitary cells can be generated as a result of loss of E-cadherin, which is responsible for the shift into the individual (single cell) migration strategy [51]. It is yet to be determined to what extent the EMT process is necessary for metastasis formation, as non-EMT cells have been shown to cooperate with EMT cells in this process [55]. Moreover, a number of studies [reviewed in 30] have demonstrated that a full EMT is not necessary for successful invasion and metastasis.

The amount of data generated on cancer progression, including in vitro studies, animal models and cancer patients, has shed new light on the complex process of metastasis formation. Better characterization of CTCs and CSCs seems necessary in order to develop new, more effective strategies of cancer eradication.

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