FEBS Letters 589 (2015) 1577–1587

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

The relevance of EMT in breast cancer metastasis: Correlation or causality?

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ARTICLE INFO

Article history:
Received 27 March 2015
Revised 6 May 2015
Accepted 6 May 2015
Available online 12 May 2015

Edited by Wilhelm Just

Keywords:
Breast cancer
Cell migration
Epithelial to mesenchymal transition
Metastasis
Mouse model

ABSTRACT

Although major progress has been achieved in treating breast cancer patients, metastatic breast cancer still remains a deadly disease. A full understanding of the process of systemic cancer cell dissemination is therefore critical to develop next generation therapies. A plethora of experimental data points toward a central role of an epithelial to mesenchymal transition (EMT) in the multistep cascade of metastasis formation. However, in patients the data are based on correlative studies which often, but not always, tie the expression of EMT markers to cancer invasion, metastasis and poor clinical outcome. Moreover, the notion that cancer cells are able to switch between different modes of migration asks for a thorough review of the actual relevance of EMT in cancer metastasis.

1. Introduction

The role of an epithelial to mesenchymal transition (EMT) as a fundamental biological mechanism is well established in morphogenic processes of the developing embryo, in wound healing and in organ fibrosis [1–3]. In addition, an EMT is frequently called upon – including by us – as a favored explanation how tumor cells gain migratory and invasive properties in order to leave the primary tumor site, to disseminate throughout the body, and eventually form distant metastases [4–6]. In the prototypical multistep model of metastasis, the function of EMT is attributed to the initial events, when tumor cells lose their epithelial characteristics to leave the primary tumor, invade into neighboring tissue and enter the blood circulation (Fig. 1). An EMT is also thought to support the survival of tumor cells in the blood stream and to promote extravasation at the distant metastatic site [7,8]. Finally, mesenchymal tumor cells that have undergone an EMT appear to share a variety of hallmarks capabilities with experimentally defined cancer stem cells (CSC; for an in depth review of the link between EMT and CSC see references [1,9]. Since mesenchymal carcinoma cells are thought to proliferate at reduced rates and since many carcinoma metastases display the same degree of differentiation as their primary tumors, it is thought that mesenchymal, invasive cancer cells undergo a mesenchymal to epithelial transition (MET) after extravasation in distant organs to form overt (macro)metastases [10,11].

The highly complex process of EMT is busily studied at the molecular level. It appears that EMT (and potentially with it metastasis) does not rely on additional genetic alterations in the cancer cells. Rather complex regulatory circuits involving transcriptional and epigenetic control mediated by distinct "EMT" transcription factors, miRNAs and lncRNAs seem to govern an EMT [8,12,13]. Despite or because of the recent insights, it is worthwhile to take a step back and ask to what extent signs of an EMT are detected in primary tumors, whether an EMT is actually required in the process of metastasis, and to discuss potential alternative models of cancer cell dissemination. Here, we focus on breast cancer, since this cancer type is frequently studied in metastasis research, mainly due to the availability of a variety of valuable transgenic and transplantation mouse models of metastatic breast cancer [14]. In addition, based on the recent molecular classifications of breast cancer subtypes and the identification of a claudin-low subtype exhibiting an EMT gene expression signature, breast cancer specifically qualifies to assess the role of EMT in the metastatic process [15].

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http://dx.doi.org/10.1016/j.febslet.2015.05.002
0014-5793/

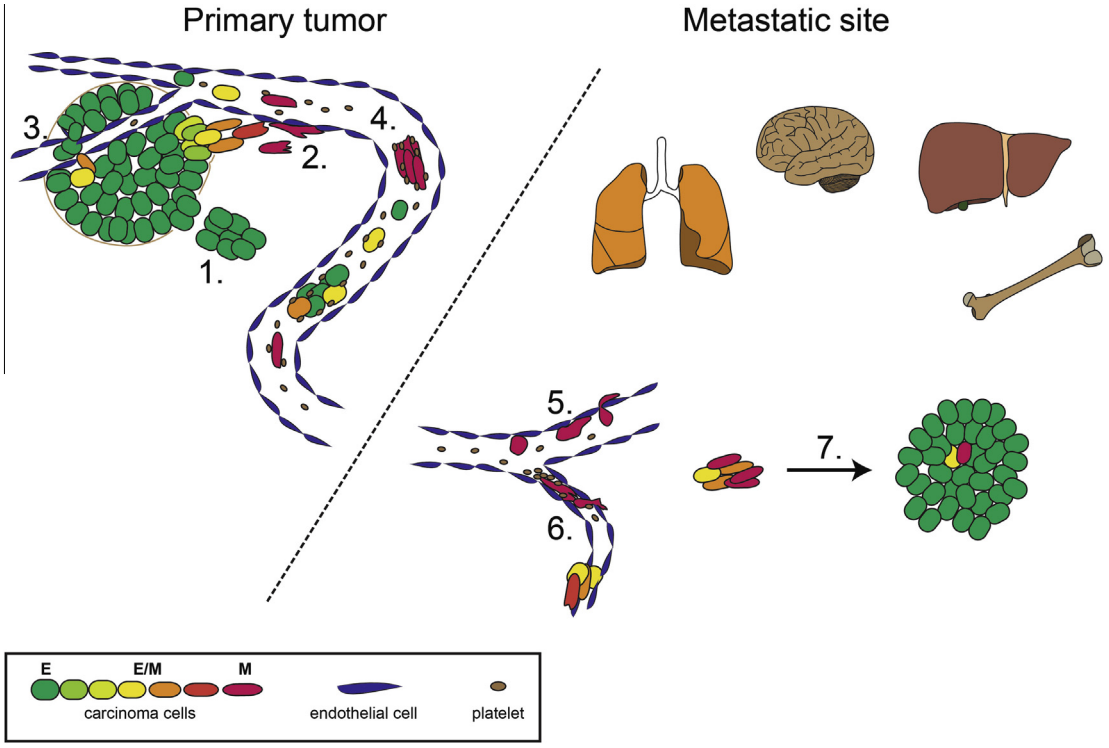

Fig. 1. The potential involvement of EMT and MET in the metastatic cascade. Carcinoma cells reach the systemic circulation by collective invasion (1) or single-cell migration of EMT-derived mesenchymal cells (2) into the blood vessels. Alternatively, they can be passively shed (3) into the blood stream. Circulating tumor cells (CTC), either single cells or CTC-clusters, are found to express predominantly a spectrum of epithelial markers (E), co-express epithelial and mesenchymal markers (E/M) or to express predominantly mesenchymal markers (M; 4). CTCs are frequently covered by platelets, facilitating carcinoma cell extravasation. At distant organ sites, surviving CTCs are potentially extravasating similar to leukocytes by initial transient contacts, followed by firm adhesion to endothelial cells and subsequent diapedesis and active extravasation, although direct proof for this multistep mechanism is still lacking (5). CTCs are also physically trapped due to size restriction in small vessels and initiate proliferation inside the vessel lumen (6). In order to colonize, i.e. to grow from micro- to macrometastases, mesenchymal carcinoma cells may need to undergo an MET (7). The hematogenous spread of breast cancer cells displays specific tropism to lung, brain, liver and bone.

2. EMT and its associated features

Carcinomas, i.e. malignant cancers of epithelial origin, often retain – until a certain state of dedifferentiation – a sheet-like morphology with apico-basal polarity and intact tight and adherens junctions. A prototypical EMT of these cells involves a spectrum of processes having in common the loss of apico-basal polarity and the delocalization of tight and adherens junction proteins, such as E-cadherin, ZO-1, occludins, and claudins. At the same time, they assume a spindle-shaped, mesenchymal-like morphology with upregulated expression of mesenchymal markers, such as N-cadherin, fibronectin and vimentin, and increased migratory and invasive properties [16]. EMT can be easily induced in breast cancer cells in 2D in vitro culture, for example by transforming growth factor (TGFβ) or the overexpression of EMT-inducing transcription factors such as TWIST. In addition, EMT-associated migratory and invasive capacities can conveniently be studied in vitro by quantifying the efficiency of cells to migrate through porous membranes, either uncoated (for migration) or coated with a layer of extracellular matrix proteins (for invasion) [17,18]. Although this reductionist approach has provided major mechanistic insights into the principles of EMT and cell invasion, the results cannot be simply extrapolated to the in vivo situation in animal models or in patients [19–22]. Studying EMT in vivo mostly relies on a retrospective, "snapshot" analysis of surrogate markers for cell migration and invasion and thus lacks critical information on the dynamic changes underlying an EMT process. Migration and invasion per se can only be visualized by technically challenging intra-vital life cell imaging techniques in 3D matrices and in living animals [23–25].

3. Cell migration, invasion and intravasation

3.1. Individual cell migration

Tumor cells migrate either as single cells (individual migration) or as multicellular groups (collective migration) [26,27]. The characteristics of a special type of cell migration, where cells are aligned in single-cell chains in so called "indian files", will be presented below when discussing the relation of EMT to the lobular histopathologic subtype of breast cancer [21]. Individually migrating cells usually employ mesenchymal traits of migration with integrin-mediated cell–extracellular matrix (ECM) adhesion dynamics characterized by the generation of high traction forces, the use of proteases for ECM cleavage and the formation of focal contacts at sites of integrin clustering [21]. As an alternative, cancer cells may "squeeze" through tissues by amoeboid migration, which is characterized by propulsive cytoplasmic forward flow, the lack of integrin-ECM contacts and the absence of proteolytic cleavage of the ECM. Notably, amoeboid migration is substantially faster than mesenchymal migration [28–30]. In addition, a hybrid amoeboid/mesenchymal phenotype of cancer cells has been described [31]. In a seminal study, the Sixt laboratory has demonstrated that murine leukocytes, which usually utilize integrin-mediated contacts to move on 2D surfaces, do not depend on adhesion to the ECM via integrins when migrating through a 3D environment in an amoeboid-like fashion [32]. In line with this finding, blockade of integrin function induces a so-called mesenchymal to amoeboid transition (MAT) even in cancer cells of solid tumors [33,34]. Cells induced to undergo an EMT by TGFβ also switch to a faster amoeboid migration mode in experimental

conditions of high confinement and the absence of matrix adhesion [28]. Numerous MAT-inducing mechanisms have been identified in the past years, including inhibition of ECM-degrading proteases or of Rac1 activity, induction of RhoA activity, the forced expression of EphA2 or p27, and p53 deficiency [21,35–40]. These reports may explain why anti-cancer therapies targeting protease or integrin functions have shown disappointing results in clinical trials [40–43].

A novel mechanism of cell extrusion has been recently proposed by which epithelial cells may leave the epithelial sheet [44]. In normal epithelial tissue homeostasis, dying cells are actively extruded apically into the lumen to preserve a tight barrier function. In contrast, oncogenic signaling in transformed cells leads to a basal extrusion of cancer cells. Since in some circumstances cancer cells can cross basement membranes (BM) without proteolytic degradation, basally extruded cancer cells might not necessarily leave behind a BM defect [26,44–47]. Whether a basal extrusion process plays a role in cancer cell dissemination and whether there is functional connection with an EMT will be part of exciting future research [44].

3.2. Collective cell migration

Collective cell migration is characterized by the simultaneous movement of a group of cells with intact cell–cell interactions. Depending on their morphological appearance, these collectives can be classified into "clusters", "strands", "tubes" or "sheets" [48]. Collective cell migration is proposed to be a predominant mode of local cancer cell invasion especially in differentiated carcinoma [49]. Leader cells guide the multicellular aggregate by proteolytic degradation of the ECM in the front and by dragging the cells of the inner and trailing edge. Since the leader cells appear to play a dominant role in the movement of these collectives, their characteristics warrant attention – also in relation to EMT. Notably, the importance of a basal epithelial program in the invasive phenotype of locally invading breast cancer has been reported [49]. Leader cells express basal epithelial markers, such as cytokeratin 14 and p63, while lacking evidence for EMT-associated features, such as the loss of E-cadherin function or increased TWIST, SNAIL or vimentin expression. Although a complete EMT seems not obvious in cells leading collectively invading multicellular groups, this does not exclude a sub-threshold level EMT or intermittent bursts of EMT, as earlier discussed by Friedl and colleagues [26]. In addition to the perception that collective migration is used by epithelial cancer cells, it is also employed by mesenchymal cancer cells [23,50–52]. In mesenchymal cells, cell–cell contacts are mediated by N-cadherin, a cadherin family member characteristic of a mesenchymal cell phenotype [51,53].

Similar to what has been described for plasticity of individually migrating cells, collectively migrating cells can eventually leave the group and continue their march individually, by either migrating in a mesenchymal or in an amoeboid mode – the latter is known as collective to amoeboid transition (CAT). Fibrosarcoma and melanoma cell lines preferentially use collective migration in 3D collagen densities (smaller ECM pore sizes), whereas lower collagen densities (bigger ECM pore sizes) induce the break-out of single cells [23], and blocking β_1-integrin induces a CAT in primary melanoma explants [50].

3.3. Cancer cell intravasation

Cancer cells can actively enter the systemic circulation employing the migration and invasion modes described above. However, one should be aware of the fact that cancer cells are passively shed into the blood stream at impressively high numbers [54–58]. In addition, not only "how" but also "where" cancer cells enter the blood circulation matters. Most investigations and deliberations on the mechanisms of cancer cell invasion, as individually or collectively migrating cells, are focusing on the invasive front, i.e. the zone of direct contact between the tumor cells and the surrounding desmoplastic stroma [59]. However, tumor cells can disseminate at the stage of carcinoma in situ as proposed by the parallel progression model possibly even before the occurrence of an angiogenic switch [60]. The significance of the invasive front might therefore primarily be a surrogate of the intrinsic invasive capacity of a tumor plus having a causal role in the loco-regional spread of cancer cells. On the other hand, tumors are highly vascularized and contain a "highway" of hematogenous spread inside the tumor mass. Indeed, intra-vital imaging has visualized the intravasation of tumor cells within the tumor mass [61]. Interestingly, an EMT drives the expression of a set of pro-angiogenic genes, partially explaining the enhanced tumor-initiating capacity often associated with the EMT process [62,63]. Based on these findings it appears that an EMT not only renders tumor cells more capable of migrating toward close-by blood vessels, but their pro-angiogenic activities may enable them to "path their own way" into the systemic circulation.

The possibility of switching between different types of cell migration illustrates the large plasticity of cancer cells and the complexity of their therapeutic targeting. The molecular mechanism underlying the different types of cancer cell migration, the conversion between these different types, the environmental factors promoting this transitions, and the characteristics of the subsets of cells of a given tumor that hold this plasticity still need to be further elucidated. The different modes of cell migration do not seem to be mutually exclusive for a given cell. In a "tuning model", the mode of cell migration is characterized as a continuum and is the result of the integration of physical and biochemical influences of the tissue environment with the genetic and epigenetic makeup of a given cell [48]. Given the emerging picture of intra-tumoral heterogeneity in cancer [64,65], it is likely that different areas within an individual tumor rely on distinct modes of cell migration and invasion.

4. Does an EMT occur in primary tumors?

Carcinomas often elicit a desmoplastic reaction with abundant mesenchymal stroma cells, such as cancer-associated fibroblasts, with predominantly pro-tumorigenic activities [66]. Conventional histopathological analysis can conveniently discriminate between epithelial cancer cells and fibroblasts with their prototypical spindle-shaped morphology. However, once epithelial cancer cells have converted to a mesenchymal morphology by an EMT, they are hardly distinguishable from stromal fibroblasts. Also the marker repertoire of cancer cells changes to a mesenchymal phenotype after undergoing an EMT and, thus, mesenchymal carcinoma cells remain indistinguishable from stromal fibroblasts by molecular or immunohistochemical analyses. Notably, the expression of cytokeratins or epithelial cell adhesion molecules such as EpCAM, which are routinely used to identify tumor cells of epithelial origin, is lost during an EMT [67]. In this context it also should be noted that, in contrast to early embryonic developmental processes, the concept of EMT in malignant tumor progression reflects a transition within the same lineage and does not signify a real conversion of cells of an epithelial lineage to a mesenchymal lineage – a distinction that has brought some controversy into the discussion about the existence of EMT in cancer [22,68].

4.1. EMT in preclinical breast cancer mouse models

Syngeneic and xenogeneic transplantation models of breast cancer cell lines in mice have been extensively used in order to establish a causal relationship between EMT and metastatic dissemination by interfering with critical mediators of EMT, including EMT-inducing growth factor signaling or transcription factor activities [2]. Although transplantation models of primary cancer cells and established cancer cell lines offer important mechanistic insights into the metastatic cascade, there value is limited by the lack of a slow co-evolution of the implanted cancer cells with the host stroma. In the case of xenografts, the differentiation state of the cells lines used, the lack of an intact immune response, and potential species incompatibilities of growth factor signaling may obscure the processes active in patients [69]. For example, the human breast cancer cell line MDA-MB-231, a frequently used xenograft metastasis mouse model, stably displays mesenchymal traits at baseline, which limits the study of the dynamic processes of an EMT [70]. Hence, to delineate a possible causal role of EMT in breast cancer metastasis we will focus on data derived from transgenic mouse models of breast cancer.

In transgenic mouse models of breast cancer, first lineage-tracing experiments have provided evidence that EMT exists in vivo. By genetically tagging tumor cells combined with immunofluorescence analysis of marker expression, Trimboli and colleagues identified carcinoma cells with a loss of E-cadherin and gain of fibronectin expression. Interestingly however, carcinoma cells with signs of EMT were only detected in a c-MYC-driven transgenic mouse model of breast cancer and not in the MMTV-PyMT and MMTV-Neu transgenic mouse models of breast cancer, which are two widely used models to study breast cancer metastasis [71]. In contrast, phospho-SMAD2 and phospho-SMAD3 have been identified in certain areas of MMTV-PyMT tumors as an indicator of active TGFβ signaling, yet the EMT marker status of these tumors has not been assessed [72,73]. The conditional deletion of p53 in mammary epithelial cells of mice (achieved by Cre recombinase expression under the control of the K14 or WAP promoters, respectively) provoked the formation of some tumors with carcinosarcomatous morphology, with heterogeneous expression for the luminal marker cytokeratin 8 and the basal marker cytokeratin 14, and with increased vimentin expression. Yet, despite the invasive phenotype of the tumors, distant metastasis is a rare event in these models [74,75]. When CRIPTO-1, a member of the epidermal growth factor-CFC protein family, was conditionally overexpressed in mammary epithelial cells, tumors eventually developed with a latency of 14–18 months in a proportion of multiparous mice. Whereas most lesions displayed a differentiated morphology classified as papillary adenocarcinomas, some tumors contained areas with an EMT phenotype (negative for E-cadherin and positive for N-cadherin, fibronectin, α-smooth muscle actin, vimentin, and SNAIL) [76]. Similarly, conditional overexpression in the adult mammary epithelium of the sine oculis homeobox 1 homolog (SIX1) homeoprotein led to tumors of a variety of different grades of differentiation [77]. A subset of the tumors displayed a sarcomatoid phenotype with the expression of markers suggestive of a complete EMT. In addition, 80% of the non-sarcomatoid tumors showed a partial EMT with areas of E-cadherin loss and nuclear β-catenin accumulation colocalizing with high expression of the Wnt target gene cyclin D1.

For a reality check as to whether transgenic mouse models of breast cancer faithfully recapitulate the patient situation, gene expression profiles of the model tumors have been compared to the gene expression of the various patient breast cancer subtypes (see also below). Whereas tumors of a variety of mouse models displayed a "luminal-like" gene expression profile (the majority of tumors from MMTV-PyMT, MMTV-Neu and WAP-Myc mice), a proportion of model tumors showed either strong expression of mesenchymal features or mixed expression of luminal, basal and mesenchymal signatures (tumors from $Brca1^{fl/fl}$;TgMMTV-Cre;$p53^{+/-}$, WAP-Myc, or DMBA-treated mice) [15].

Taken together, there is convincing evidence that tumor cells bearing a mesenchymal phenotype exist in primary tumors of transgenic mouse models of breast cancer. To assess whether even rare cells with mesenchymal features, which are unable to significantly influence the global gene expression profile of the bulk of a tumor, are present in the metastatic mouse models of breast cancer, appropriate lineage-tracing experiments need to be performed. Such genetic fate mapping of tumor cells combined with immunofluorescence staining for mesenchymal markers has recently identified an EMT as a very early event in a pancreatic ductal adenocarcinoma mouse model [78].

4.2. Mechanisms of EMT in mouse models of breast cancer

It is important to note that any evidence for an EMT in a primary tumor does not necessarily allow the conclusion that an EMT is a prerequisite for the metastatic process. To this end, functional studies are needed in which "key EMT players" are genetically manipulated in transgenic mouse models of breast cancer. Subsequent characterization of changes in EMT marker expression in primary tumors and metastatic lesions, the assessment of primary tumor grade and local invasiveness as well as the metastatic burden (typically in the lung) reveals the functional roles of factors of interest in EMT and/or metastasis. Candidates to be assessed could be EMT-inducing cytokines, such as TGFβ, EGF, FGF, and HGF, hypoxia induced by rapid tumor growth or by the pharmacological inhibition of blood vessel angiogenesis (anti-angiogenic therapies), components important for cell–cell contact and cell polarity, and EMT-inducing transcription factors, such as TWIST, SNAIL1/2, and ZEB1/2 [2,16]. Exciting insights have already been obtained by studying the tumor-promoting role of TGFβ and some of the transcription factors relevant for EMT (see below), yet further studies are needed to identify and distinguish between "simple" markers of an EMT in vivo and factors with non-redundant functions during an EMT.

TGFβ, one of the best-studied EMT-inducing cytokines, is produced by both tumor cells and by a variety of cells of the tumor microenvironment. It exerts important effects on several cell types within a tumor and by canonical, SMAD-dependent and non-canonical, SMAD-independent signaling modulates the expression of a variety of target genes to either exert tumor suppressive functions, such as induction of the cell cycle inhibitor p21 or repression of c-MYC, or tumor-promoting functions by inducing an EMT (for a detailed description of TGFβ signaling and its role in cancer see reference [79]). Consistent with this notion, MMTV promoter-driven mammary epithelial cell-specific expression of TGFβ in the MMTV-Neu model results in primary tumors with higher tumor grades and increased metastatic burden in the lungs. Interestingly, despite the TGFβ-mediated increased local invasiveness, primary tumors still express E-cadherin and do not upregulate the mesenchymal markers vimentin, alpha-SMA and fibronectin [80]. Similarly, overexpression of TGFβ in MMTV-PyMT transgenic mice at late stages of tumor development dramatically increases metastasis to the lungs [81]. In line with the pro-metastatic activities of TGFβ in the MMTV-Neu driven breast cancer mouse model, expression of a constitutive–active TGFβRI promotes the metastatic process, whereas expression of a dominant-negative TGFβRII inhibits lung metastasis [82,83]. Notably, one of these studies has revealed an important role of TGFβ signaling for tumor cell extravasation rather than for primary tumor invasion and tumor cell intravasation [83].

Encouraging data from a therapeutic point of view comes from experiments where neutralization of TGFβ by a soluble TGFβRII:Fc fusion trap has reduced the incidence of lung metastasis in MMTV-Neu and MMTV-PyMT mice [84]. Since the TGFβRII:Fc trap reduced the number of colony-forming circulating tumor cells (CTCs), the authors suggested a role for TGFβ in intravasation. Alternatively however, CTCs could also come from growing metastases, and the reduction in CTCs may simply reflect the lower metastatic burden. In contrast to these reports, the Moses laboratory has reported a metastasis-promoting effect when attenuating TGFβ signaling in the MMTV-PyMT and MMTV-Neu models by a tumor cell-specific deletion of TGFβRII or the expression of a dominant-negative TGFβRII, respectively [72,85]. Mechanistically, abrogation of TGFβ signaling in the MMTV-PyMT model results in the recruitment of $Gr1^+CD11b^+$ myeloid-derived suppressor cells, which by secreting MMPs promote invasion of E-cadherin-positive tumor cells [86]. In TGFβ-attenuated MMTV-Neu tumors, increased VEGF-A expression provokes leaky vessels and potentially facilitates cancer cell intravasation [85]. Based on TGFβ's context-dependent and highly complex impact on tumor cells and on cells of the tumor microenvironment, it is not surprising that manipulation of the different components of the TGFβ/TGFR signaling axis leads to a wide range of sometimes contradictory effects on metastasis formation.

Employing the Rip1Tag2 transgenic mouse model of neuroendocrine carcinoma of the pancreas we have previously shown that abolition of E-cadherin (Cdh1)-mediated cell–cell adhesions can be a trigger of tumor invasion and metastasis [87]. Derksen and co-workers have recently reported that the concomitant genetic ablation of *Cdh1* and *Trp53* in mammary epithelial cells of the mouse induces the formation of invasive and metastatic lobular breast carcinomas which, however, do not display features of a complete EMT [74,75]. These data suggest that, although loss of E-cadherin is sufficient to induce local invasion and distant metastasis, it does not necessarily promote a complete EMT – in contrast to what is observed in cell culture experiments [88,89]. A characteristic molecular event observed during an EMT is the transcriptional shut-off of the *Cdh1* gene by EMT-inducing transcriptional repressors, such as SNAIL1/2, TWIST1/2, and ZEB1/2 [16]. Accordingly, the inducible expression of TWIST1 and with it the induction of an EMT in the primary tumor site allows the dissemination of tumor cells to distant organs. However, metastatic outgrowth at the distant organs requires the loss of TWIST1 expression and a mesenchymal-to-epithelial transition (MET; see below; [90]). In a mouse model of invasive mammary carcinoma with doxycycline-inducible expression of a constitutive–active version of the Her2/Neu (NeuNT) oncogene, NeuNT-driven tumors completely regress after doxycycline withdrawal and oncogene expression shutdown, yet they display NeuNT-independent recurrence after a latency of several months [91]. Whereas tumors occurring during the initial NeuNT-driven growth phase show extensive lung metastasis but retain an epithelial morphology, the recurring tumors display a SNAIL1-driven mesenchymal phenotype with downregulation of CK8 and E-cadherin and upregulation of vimentin and fibronectin expression. Indeed, the experimental manipulation of a variety of EMT-relevant genes, such as the genes encoding for SNAIL1/2, TWIST1/2, ZEB1/2, FOXC2, SOX4, TEAD2, LHX2, and others, results in a change in breast cancer cell invasion and dissemination and in metastasis formation, evidencing a link between EMT and the disseminating and tumor-initiating capabilities of carcinoma cells (reviewed in [16,92,93]). However, a formal proof that metastases are indeed initiated by cancer cells that have ever undergone an EMT is still lacking and will require sophisticated fate-mapping experiments in animal models.

5. Extravasation, MET and colonization

5.1. Extravasation

Disseminating cancer cells, after having survived the harsh conditions of their travel through the blood stream, have in principle two possibilities how they can form a metastatic nodule in a distant organ: physical trapping in small capillaries of the target organ due to size restriction and initial proliferation inside the vascular lumen and subsequent disruption of the vessel wall as the metastasis expands [94], or extravasation and subsequent proliferation in the extra-luminal compartment. While the former is difficult to address experimentally without sophisticated intra-vital imaging, the latter is supported by first experimental evidence: similar to the behavior of leukocytes when egressing from blood vessels at inflammatory sites by binding to selectins and subsequent firm adhesion via integrins (tethering, rolling), cancer cells are able to establish weak contacts with endothelial cells in vitro [95]. However, in vivo evidence for the existence of such initial weak contacts is still missing [96]. Stable contacts of tumor cells to endothelial cells seem to be mediated by adhesion molecules such as members of the integrin-family and CD44 and N-cadherin [96,97]. Several of these molecules with important functions during transendothelial migration (TEM) are upregulated during an EMT. For example, increased N-cadherin expression and the loss of E-cadherin expression, the cadherin switch, is one hallmark of an EMT [53]. Moreover, EMT-induced integrins on cancer cells can interact with cell adhesion molecules (CAMs) expressed by endothelial cells. Finally, an EMT often leads to the upregulation of enzymes that modify carbohydrate moieties on selectin-binding glycoproteins. On one hand, CD44 isoforms with specific glycosylated residues have been described to mediate initial weak contacts by binding to E-selectin expressed by endothelial cells, whereas on the other hand CD44 plays an important role by mediating firm adhesion to endothelial cells [96]. Notably, human breast cancer cells that have undergone an EMT and resemble breast cancer stem cells are characterized by the expression of high levels of CD44 [98]. In addition, upregulation of TGFβ and VEGF-A by an EMT can enhance permeability of the capillary bed and thereby promote TEM [99,100]. Hence, the pro-angiogenic phenotype of cancer cells acquired during an EMT does not only promote intravasation by creating a disorganized vessel network in the primary tumor but also may facilitate extravasation at distant sites. The extravasation process involves a crosstalk between the cancer cells and endothelial cells, and leukocytes, platelets and proteins of the coagulation cascade play supportive roles [7,101]. For example, platelets frequently embrace circulating tumor cells and, by releasing TGFβ, they induce an EMT of cancer cells inside the blood stream and thus increase their extravasation into the lung [7]. Amoeboid cell migration of cancer cells, on the other hand, has also been shown to promote TEM [102]. However, the spatial and temporal contribution of the mesenchymal vs. the amoeboid phenotype during the process of TEM and subsequent crossing of the underlining basement membrane during the process of cancer cell extravasation in vivo has to be further investigated.

5.2. MET and colonization

If EMT plays an important role in metastasis formation, how comes that carcinoma metastases frequently display a similar degree of differentiation as the primary tumor [11]? One formal possibility has it that EMT is dispensable for intravasation, and cancer cells rather intravasate by collective migration of epithelial cell clusters or by passive shedding into the circulation, promoted by the disorganized and leaky vasculature present in the primary

tumor [49,103]. Another explanation is based on the notion that EMT is a transient/dynamic process conferring high plasticity to cancer cells. Thereby it has been postulated that EMT-derived mesenchymal cells, which are thought to be slowly proliferating cells with cancer stem cell-like properties, are forced to undergo a mesenchymal to epithelial transition (MET) to be able to initiate proliferation [11]. If so, what triggers an MET? Is it simply the lack of EMT-inducing factors in the inhospitable environment of metastatic target organs, or are distinct factors actively promoting MET?

There is a plethora of experimental evidence, supporting the hypothesis that MET in the target organ is required for colonization. One of the earliest reports about the requirement for MET during metastatic outgrowth comes from experiments with a human bladder carcinoma cell line, which by serial passaging in mice gave rise to subclones with increased metastatic potential. Whereas the parental cell line displayed mesenchymal features, the higher metastatic subclones were of epithelial morphology and expressed epithelial markers. However, in contrast to these results by intracardial injection, when the cells were injected orthotopically and all steps of the metastasis cascade had to be successfully completed to form metastases, the mesenchymal parental cell line has shown higher metastatic potential than the epithelial subclones. These results raise the possibility that an EMT plays a critical role in the early steps and an MET in the late steps of the metastatic cascade. Mechanistically, MET can be promoted by increased FGFR2 expression, interestingly by the mesenchymal-specific splice isoform FGFR2IIIc [104]. In line with these findings, cells isolated from lung metastases of patient-derived breast cancer xenograft mice (PDX mice) of the basal-like subtype partially lose their aggressiveness compared to the parental tumor cells, accompanied by a more differentiated, MET-like status [105]. A requirement for MET in lung colonization has been further demonstrated by spatially restricting TWIST1 expression to the primary tumor site and preventing its expression during the lung colonization process [90] or by reducing the expression of the EMT-inducing transcription factor PRRX1 [106]. Along these lines, mesenchymal, E-cadherin-deficient breast cancer cells derived from the MMTV-Neu mouse model seed more lung metastases upon orthotopic mammary fat pad injection as compared to epithelial, E-cadherin-expressing cells. However, mesenchymal, E-cadherin-deficient cells are less metastatic as compared to the epithelial cells when injected into the tail vein [62], again supporting a critical role of an EMT in the early and of an MET in the late stages of metastasis formation. Although TGFβ is a well-characterized inducer of EMT, a recent report has proposed that TGFβ can also induce MET in mesenchymal cancer cells via induction of ID1, which in a dominant-negative manner inhibits TWIST1 activity and increases stem cell-like features of the cells [107]. The same publication proposes the existence of both epithelial and mesenchymal cancer cells with tumor-initiating properties. Indeed, two distinct populations of cancer cells in primary breast cancer patient samples have been identified: an epithelial cell population expressing the enzyme aldehyde dehydrogenase and a mesenchymal cell population characterized as $CD44^{+}$ $CD24^{-}$ [108]. In contrast to the reports above underlining the importance of MET in metastatic colonization, recent work has demonstrated that a mesenchymal phenotype with increased β1 integrin and focal adhesion (FAK) activity and increased filopodia formation promotes active lung colonization and metastatic outgrowth of breast cancer cells [95,109]. Moreover, overexpression of a constitutive–active form of the EMT-inducing transcription factor TEAD2 promotes rather than inhibits lung metastasis formation upon i.v. injection of murine breast cancer cells [92]. Apparently, the fine-tuned cell plasticity and the functional interplay between EMT in earlier stages and MET in the later stages of the metastatic cascade warrants further investigation.

6. Evidence for EMT in human breast cancers

Breast cancers do not represent a single cancer entity but instead summarize a broad spectrum of different malignant diseases of the breast. Several classification systems are employed in order to provide the optimal treatment regiment for breast cancer patients. Traditionally – and still of great value – breast cancers have been classified according to their morphologic appearance into several histological types. Secondly, immunohistochemical analysis of the estrogen receptors (ER) and progesterone receptors (PR) as well as HER2 expression stratifies patients to anti-hormonal therapy and therapeutics targeting HER2. Since the beginning of this millennium, a new layer of classification has been achieved by the use of gene expression profiling [110,111].

In the early days, pathologists have developed and employed a highly sophisticated classification system for malignant diseases of the breast based on cancer (cell) morphology. Most invasive breast cancer patients are diagnosed with *invasive breast carcinoma of no special type* (previously termed invasive ductal carcinoma) [112]. Two less frequently observed subtypes, which are interesting with regards to EMT, are *invasive lobular carcinoma* and *metaplastic carcinoma. Invasive lobular carcinoma* cells can migrate as so-called "indian files", which is regarded as a single-cell migration mode, although the cells are in close contact to each other at their front and rear [27]. One hallmark of *invasive lobular carcinoma* is the mutation or reduced expression of E-cadherin. Interestingly, based on gene expression analysis, these tumors were highly prevalent in the luminal A subtype, which is usually characterized by its well-differentiated epithelial morphology [113]. These data, together with the results from the E-cadherin-deficient mouse models of lobular breast carcinoma discussed above [75], contradict the results from in vitro experiments demonstrating that a loss of E-cadherin function is sufficient to induce a complete EMT [62,89].

Metaplastic *carcinomas* represent – among others – tumors with mesenchymal phenotype and they are typically negative for ER, PR and HER2 expression (i.e. triple-negative). For the nomenclature of the mesenchymal representatives of this subtypes, the terms carcinosarcoma and sarcomatoid carcinoma are often used as synonyms. Interestingly, metaplastic carcinomas are often classified as basal-like or claudin-low (identified based on an EMT-like gene expression signature, see below), they frequently carry mutations of *TRP53* and generally respond less to chemotherapy than other triple-negative breast cancers [70,114]. Regarding the etiology of their mesenchymal appearance, it is currently unclear if this is due to epigenetic (potentially reversible EMT) or genetic (irreversible EMT) alterations. Other than that, using the term of an EMT to describe the etiology of their mesenchymal morphology implies an epithelial cell as origin, a notion that currently lacks experimental support.

The advent of gene expression profiling has revolutionized the classification systems of cancer in general and of breast cancer in particular and has revealed functional insights into the biological processes underlying breast cancer morphology. Based on gene expression profiling, invasive breast cancers were initially classified into the intrinsic subtypes luminal A, luminal B, HER2-enriched basal-like, and normal breast-like [110,115]. Later on, these categories were extended by the claudin-low subtype, which with regard to EMT warrants further attention [15,70].

The claudin-low breast cancer subtype is characterized by the low expression of epithelial markers (E-cadherin, occludin, claudin

3, 4 and 7), luminal markers (cytokeratins 18/19, GATA3), and the receptors ER, PR, HER2 (triple-negative). It is distinct from the closely related triple-negative, basal-like subtype – besides the enrichment of an EMT gene expression signature – by lower expression of proliferation genes [70]. On the other hand, claudin-low tumors display increased expression of genes involved in angiogenesis, cell migration, immune system response (i.e. CXCL12) and extracellular matrix (vimentin), to name but a few. Overall, the gene expression profile of claudin-low tumors suggests an EMT phenotype with a significant amount of different infiltrating leukocytes [70]. Importantly, several studies have shown that tumors of the claudin-low, but not of the basal-like subtype, are enriched in cancer stem cell/tumor-initiating cell signatures, which is consistent with the finding that EMT and tumor-initiating properties are often shared [1,70]. Regarding their histopathological appearance, of the tumors classifying as claudin-low most of them classify as *invasive carcinomas not otherwise specified*, while only a minority is characterized as *metaplastic or medullary carcinoma* [70].

If EMT plays an essential role in the process of leaving the primary tumor and entering the systemic circulation, one would assume that the higher the percentage of cells with an EMT phenotype in the primary tumor is, the higher the likelihood of distant metastasis and shorter patient survival will be. However, an EMT signature does not predict breast cancer patient survival [116]. Moreover, claudin-low tumors do not show a worse prognosis than luminal B, HER2-enriched or basal-like – the other subtypes with poor prognosis [70]. These results are rather surprising, since it has been shown for many individual key EMT players, including FOXC1, SOX4, LXH2, PRRX1, and for a signature composed of TGFβ-pathway components and downstream targets that their high expression correlates with poor clinical outcome [5,93,106,116,117]. An association between lung metastasis relapse and an enrichment of a TGFβ-response signature has only been found in ER^- primary breast tumors but not in ER^+ breast tumors, and this signature is not prognostic for metastatic relapse in the liver, bone and brain [100]. In contrast to the lack of prognostic impact on patient survival, several reports have linked tumors of the claudin-low subtype with resistance to chemotherapy, in concordance with the general assumption that cells with an EMT phenotype are intrinsically more refractory to chemotherapy [118]. Along these lines, a gene expression signature representing stromal cells or mesenchymal tumor cells has been associated with a poor response to neoadjuvant chemotherapy [119]. In addition, claudin-low tumors have shown to be less chemosensitive than basal-like tumors [70], and a pathological complete response has been negatively correlated with an EMT signature [116]. Intriguingly, the claudin-low and a cancer stem cell signature are enriched after neoadjuvant treatment with endocrine therapy or chemotherapy compared to pre-treatment conditions [120]. Taken together, it appears that the claudin-low signature per se is not an indicator of poor prognosis compared to other aggressive intrinsic breast cancer subtypes, but it seems to be predictive for inferior response to therapy.

Many question remain. For example, similar to the case of metaplastic tumors discussed above, why do certain breast tumors display a mesenchymal gene expression profile? Is it due to genomic alterations resulting in an irreversible EMT or is it rather due to less stable epigenetic marks or constant EMT-inducing signals of the surrounding tumor stroma? In addition, if the cell of origin of claudin-low tumors is found within the mammary stem cell compartment, the term EMT may be misleading, since the cell of origin never has achieved an epithelial differentiation [121,122].

Global gene expression profiling of breast cancer samples has critically contributed to the understanding of inter-tumoral heterogeneity; it has provided important information about the predominant intrinsic breast cancer subtypes in the sample analyzed. However, it does not account for the potential co-existence of different tumor cell subpopulations – i.e. intra-tumoral heterogeneity [70]. Apparently, industrious single cell analysis seems required to address the extent and quality of tumor heterogeneity in breast cancer.

To potentially detect rare cancer cells with an EMT phenotype, immunostainings of tumor sections with antibodies against epithelial and mesenchymal markers have been performed. The technical hurdle to distinguish stromal cells from mesenchymal tumor cells in human tumors has recently been elegantly circumvented by performing RNA in situ hybridizations (RNA-ISH) on HER2-positive primary breast cancer patient samples concomitantly against HER2 – to identify tumor cells – and against a collection of mesenchymal markers [123]. This approach identified tumor cells expressing mesenchymal markers in these primary tumor samples. In the same study, by using dual-colorimetric RNA-ISH against a pool of epithelial transcripts and a pool of mesenchymal transcripts, they identified biphenotypic cells co-expressing both epithelial and mesenchymal markers in primary breast cancer samples – interestingly, not necessarily at the invasive front – and in draining lymph nodes. Strikingly, the highest percentage of epithelial and mesenchymal double-positive tumor cells is found in the triple-negative subtype known to display a particularly aggressive clinical course [123]. Similarly, it has been reported that cells co-expressing epithelial and mesenchymal markers are predominantly observed in samples of claudin-low and basal-like tumors [70,124]. These findings clearly show that "partial EMT" (i.e. the co-expression of epithelial and mesenchymal markers) can be observed by histopathological analysis of human breast cancer tissue. Hence, a complete EMT might not be a prerequisite for tumor cell dissemination, consistent with the observation that partial EMT represents a state with higher cell plasticity than a complete EMT [125]. Whether cells with a complete EMT can be identified within the bulk of cancer-associated fibroblasts (CAFs) within the tumor stroma remains unclear. While tumor cell-specific genetic alterations can be found in stromal cells of breast cancers [126,127], clonal somatic genetic alterations have not been found in CAFs isolated from breast and ovarian cancer stroma [128].

7. Lessons learned from circulating tumor cells (CTCs)

The prototypical role of EMT in cancer progression is often described as the initial process of the metastasis cascade, i.e. the gain of migratory and invasive properties allowing cancer cells to leave the primary tumor, to invade into nearby blood vessels and to access the blood circulation – the "highway" of cancer cell dissemination. Hence, the analysis of CTCs in cancer patients might give indirect insights into the state tumor cells are in, when they have reached the blood stream, with the caveat that CTCs can also originate from existing metastases [84]. A growing body of evidence shows that the presence of CTCs in breast cancer patients is not only associated with poor prognosis, but it is also predictive for reduced therapy response. Indeed, CTCs expressing EMT markers, such as TWIST1 and vimentin, have been identified in breast cancer patients [129,130]. One important caveat screening the literature about CTCs with an EMT phenotype is that the technically highly demanding analysis of CTCs is often biased toward the epithelial phenotype, since conventional CTC capture technologies have been frequently based on epithelial markers, such as EpCAM and cytokeratins, markers that are lost during a complete EMT [123,131]. A growing panel of new microfluidic CTC-capture devices now allows the isolation of CTCs of the whole spectrum from "fully" epithelial to "fully" mesenchymal tumor cells

[103,123,132]. Employing these devices, it has been found that the proportion of CTCs expressing various levels of mesenchymal markers is higher in more aggressive breast cancer subtypes and rises during failure of conventional chemotherapy and targeted agents [123]. The detection of CTCs expressing mesenchymal markers and its correlation with parameters of poor clinical outcome in breast cancer patients suggests therefore the importance of EMT in the intravasation process. Alternatively, EMT can also be induced after having reached the blood stream via EMT-inducing factors, such as TGFβ secreted, for example, by platelets that adhere to single-cell CTCs and CTC clusters [7,103,123]. This "outside of the primary tumor" induction of EMT may also be functionally important by preventing the CTCs from anoikis and from eradication by chemotherapy and also by supporting extravasation at the distant site.

Although CTC clusters are long known to be important contributors to metastasis formation [54], it has recently been shown that in the breast cancer transplantation models CTC-clusters represent only around 2–5% of CTCs, yet are responsible for approximately half of the lung metastases. This data reveals a dramatically higher metastatic potential of CTC clusters compared to single-cell CTCs – at least in the lung, representing the first capillary bed breast cancer cells encounter when disseminating systemically. CTC clusters are mainly derived from oligoclonal aggregates from the primary tumor rather than being generated by intravascular aggregations or intravascular proliferation [103]. Interestingly, CTC clusters can co-express epithelial and mesenchymal markers [123], raising the question by which mechanism these oligoclonal clumps have reached the systemic circulation: by collective cell migration or by passive shedding into the circulation in an epithelial state and subsequent (partial) EMT induced by platelet-derived TGFβ, or by induction of EMT within the primary tumor and passive shedding or collective migration of mesenchymal tumor cells into the blood stream? Certainly, the advances in CTC capturing technologies will not only provide important new insights into the biology of cancer cells "en route" from the primary tumor to distant sites [103], but also open new avenues for new strategies to interfere with metastasis formation [133].

8. Concluding remarks

There is compelling evidence for the existence of carcinoma cells with a mesenchymal phenotype in human breast cancer as well as in mouse breast cancer models. Sophisticated lineage-tracing experiments as well as novel technologies in single cell analysis will further shed light into the question whether rare EMT-derived mesenchymal cells can be found in the tumor stroma. However, the simple presence of EMT in the primary tumor does not allow the conclusion that EMT is actually required for metastasis. The highly complex multistep metastasis cascade and the transient nature of EMT render it difficult to draw causal conclusions regarding the importance of EMT for metastasis formation in cancer patients. In addition, mesenchymal migration represents just one of multiple migration modes cancer cells can employ to leave tissue boundaries, and therapeutically interfering with mesenchymal migration might activate salvage pathways, such as MAT, or reactivate dormant, mesenchymally disseminated tumor cells by inducing an MET.

The functional manipulation of key EMT players in breast cancer mouse models has provided clear evidence for a causal involvement of EMT-inducing or blocking factors in metastasis. Unfortunately, EMT marker analysis of the primary tumors derived from these functional experiments has been rarely reported, and whether EMT is indeed a prerequisite for metastasis formation remains to be resolved. In addition, the transient nature of EMT adds another layer of complexity to interpreting the data derived from these experiments. While temporal resolution can be achieved by the inducible expression or silencing of genes of interest, spatial resolution as performed by Tsai and co-workers is urgently needed as well [90].

Despite the impressive progress in the past years, we still need to learn about the mechanisms underlying cancer metastasis in mice and men. Animal models that closely recapitulate the patient situation and the careful design of meaningful clinical studies accompanied with cutting-edge translational research programs will be instrumental to transform cancer from a deadly into a chronic or even curable disease.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank N. Meyer-Schaller for critical input into the manuscript. Related research in the authors' laboratory has been supported by EU-FP7 TuMIC HEALTH-F2-2008-201662, the SystemsX.ch RTD project Cellplasticity, the Swiss National Science Foundation and the Swiss Cancer League. We apologize to all colleagues whose important work we could not cite due to space limitations.

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