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The Landscape of Circulating Tumor Cell Research in the Context of Epithelial-Mesenchymal Transition

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Circulating tumor cells · Epithelial-mesenchymal transition, associated phenotypes · Metastasis · Dissemination · Prognostic markers · Predictive markers

Abstract
The metastatic spread of cancer accounts for the vast majority of cancer-related deaths. It is mediated by tumor cells circulating in blood (called circulating tumor cells, CTCs), which escaped from their established niches. CTCs give a unique opportunity to look into the metastatic cascade and to study the molecular processes supporting the spread of tumor cells throughout the body. As current therapies are not sufficiently effective in treating metastatic disease, it is important to determine cellular and molecular features of cancer cells that "seed" new tumors in distant organs at early stages. In this review we focus on the role of the epithelial-mesenchymal transition program in providing a survival advantage to metastasizing tumor cells, especially CTCs, and put it in the context of clinical findings.

Introduction

The presence of cancer cells in blood corresponds to one of the first steps of the metastatic cascade, which is a complex, multistep process that requires cancer cells to detach from the primary tumor, migrate through adjacent tissue, access and travel through the vasculature (as circulating tumor cells, CTCs), and then survive and proliferate in distant organs [1]. In some cancer cells, which arise from polarized and nonmotile epithelial cells, a migratory phenotype develops under the influence of various stroma-derived cytokines leading to reactivation of the embryonic program of epithelial-mesenchymal transition (EMT) [2, 3]. During the traverse from the primary tumor to distant sites, cancer cells leave a supportive niche, which they have shaped for many years of tumor development, and enter a hostile milieu. Numerous data from in vitro and in vivo studies indicate that EMT is switched on in metastasizing cancer cells as it allows them to overcome limitations imposed by the environment. In this review we would like to summarize the current knowledge from basic and clinical studies regarding the role of epithelial and mesenchymal phenotypes of cancer cells, especially CTCs, in cancer progression.

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Role of EMT in CTC Biology

CTCs have been considered the direct mediators of metastasis, and their detection in a number of solid tumors correlates with poor prognosis [4–13]. They were found more frequently in patients with metastatic cancer than in early setting [14]. But only recently has the evidence for their metastatic potential been found in in vivo studies. Metastasis-initiating cells capable of forming metastasis to bone, lung and liver metastases in mouse xenografts were found among CTCs isolated from breast cancer (BC) patients [15, 16], confirming the hypothesis that CTCs are the real seeds of metastasis according to Paget's seed and soil theory [17]. The number of EpCAM+CD44+CD47+MET+ CTCs correlated with lower overall survival (OS) and increased number of metastatic sites [15]. Another study identified EpCAM-negative CTCs as capable of generating brain and lung metastases when xenografted in nude mice [16]. A potential signature of brain metastasis comprising "brain metastasis selected markers" HER2+/EGFR+/HPSE+/Notch1+ was identified in those EpCAM-negative CTCs [16].

Interestingly, in the study of Mego et al. [18] it was already noted that in metastatic BC (MBC) patients with undetectable epithelial CTCs (using CellSearch®) aggressive cancer subtypes and brain metastases are more frequent. Further studies showed that mesenchymal CTCs are detected in 41% of MBC patients, especially in patients with triple negative or HER2+ tumors, where mesenchymal CTCs are the major subpopulation of CTCs [19]. Patients whose CTCs expressed EMT markers had a worse prognosis than patients with fully epithelial CTCs or no CTCs [4, 20]. Therefore, clinical data point to asymmetry in frequency and malignancy of epithelial/mesenchymal phenotypes of CTCs, which has meaningful clinical implications.

EMT is an evolutionary conserved developmental program, which might be induced during cancer progression and contribute to metastatic spread [2, 3]. It is described that EMT endows cancer cells with metastatic properties by enhancing mobility, invasion, and resistance to apoptotic stimuli [2, 21]. Furthermore, EMT-derived tumor cells might acquire stem cell properties and exhibit therapeutic drug resistance [22]. Thus, the EMT program has emerged as a central driver of tumor malignancy, and its implications for CTC biology are under intensive investigation. Transitions between epithelial and mesenchymal phenotypes, and its reverse mesenchymal-epithelial transition (MET) are believed to be hallmarks of cellular plasticity allowing cancer cells a more efficient metastasis [23, 24]. According to this model, metastasis often begins when primary tumor cells undergo EMT, i.e. they lose adhesion with neighboring cells partially or completely and gain migratory and invasive traits, eventually entering the bloodstream as CTCs [1, 3]. Upon reaching a distant organ, these CTCs exit the bloodstream and undergo MET which is crucial for establishing a fully grown metastasis as it restores the high proliferative phenotype to cancer cells. Such a model would most probably hold true for hematogenous dissemination from the primary tumor, where cancer cells need to sequentially acquire invasive and proliferative features, but might be dispensable when metastasis takes place via another route. Metastasis via the lymphatic vasculature, which lacks a basal membrane, is devoid of pericytes, and where positive interstitial pressure is present [25], minimizes the need of tumor cells to migrate as they might be passively carried towards lymph nodes with the draining lymphatic fluid [26–28]. Thus, activation of EMT in one compartment might not provide the same advantage as in another. In experimental metastasis, where tumor cells are injected into the circulation, invasive properties characteristic of the mesenchymal phenotype are also becoming less significant, which does not render the mesenchymal phenotype fully dispensable. It is to be determined to what extent genetic manipulation used to study the role of EMT in cancer progression interferes with the physiological role of EMT, as high levels of EMT transcription factors, TWIST1/2, SNAIL, and SLUG, might downregulate crucial oncogenic switches as p53 or Rb [29–34]. Importantly, gene dosage-dependent function of *TWIST1* was observed [35], which further underlines the complexity of the process. Thus, by artificially inducing EMT transcription factors (EMT-TF) physiological transition between EMT and MET states can be affected. It can be of a great importance, as epithelial and mesenchymal features are not mutually exclusive and coexpression of markers characteristic of both states might characterize a highly plastic cell phenotype. Such a state (called epithelial-mesenchymal plasticity) could, to some extent, endow cells with certain positive metastatic features present in epithelial and mesenchymal phenotypes alone (like increased resistance to anoikis provided by the mesenchymal state and proliferative features, which are the attributes of the epithelial state) [36]. Due to the transient phenotype of such states, it is envisioned that those plastic cells might not be easily identified but could significantly contribute to metastatic outgrowth [36]. Thus, tumor cells with a fully mesenchymal phenotype (post-EMT) might represent a quiescent fraction of cancer stem cells, which requires

additional stimuli to initiate growth of distant epithelial metastases, whereas cells in an intermediate state, coexpressing epithelial and mesenchymal markers, would represent proliferating progenitor cells, able to more rapidly initiate distant colonization [37], similarly to cancer stem cells, which despite being rare are able to successfully maintain growth of an organ through life.

However, until now there has been no standard method or biomarker to define the EMT state of cells, which can lead to discordant interstudy results, when different markers are used. Cytokeratins (CKs) and vimentin are the most frequently used markers for epithelial and mesenchymal phenotypes, respectively. In addition, N-cadherin, TWIST1, SNAIL, SLUG, ZEB1, FOXC2, AKT2, PI3Kα, and fibronectin were also adopted as mesenchymal markers by a few investigators, while EpCAM (epithelial cellular adhesion molecule) and E-cadherin remained the main representatives for epithelial markers (Table 1). Cell surface vimentin was found to be a highly specific marker, expressed only on cancer cells [38]. The mechanism by which vimentin is being transported to the cell surface is not fully understood. It was shown that the process is positively regulated by phosphorylation – inhibition of cellular phosphatases results in increased presentation of vimentin on the surface of cancer cells, but not normal leukocytes [39]. It is possible that vimentin structural and functional changes caused by phosphorylation [40] result in its shift to the cell surface. In a microfluidics-based PCR system, expression profiles characteristic of mesenchymal cancer cells were identified (including *IGF1*, *IGF2*, *EGFR*, *FOXP3*, and *TGFB3*) in blood samples of prostate cancer patients [41]. Recently, plastin-3, a specific, novel marker useful for the detection of CTCs undergoing EMT, has been described [42, 43]. Also, although with a slight difference, some investigators used a ratio of epithelial and mesenchymal markers to describe CTC phenotypes [19, 44].

Cluster Formation for Efficient Metastasis

In cancers of different origins, studies in animal models and clinical samples have shown that CTCs can be detected as single cells or clusters containing two or more CTCs (also called circulating tumor microemboli) [14, 19, 45–48]. The frequency of such events estimated from mouse models showed that single CTCs dominate (97.4%) over CTC clusters (2.6%), but CTC clusters have higher metastatic potential [49, 50]. Recently CTC clusters have been shown to be 23–50 times more competent to seed metastases than single cells [45]. Clustered CTCs are most likely directly derived from the primary tumors, rather than the proliferation of single CTCs or the aggregation of circulating CTCs [45]. Deep sequencing analysis of prostate cancer cells revealed evidence for frequent polyclonal seeding from the primary tumor to different secondary sites [51], which might suggest direct seeding by multicellular CTC clusters. These clinical data are also supported by in vivo studies using multicolor lineage-tracing technology in mouse models showing that polyclonal metastases may be seeded by tumor cell clusters [45, 52, 53]. Importantly, clusters exhibit superior survival and colony-forming potential in culture [45], in vivo [45, 52, 53] and probably in the clinical setting [54]. Within cell clusters from small cell lung cancer patients subpopulations of apoptotic cells were not detected, which might implicate protection from anoikis [54].

The biological basis of metastatic seeding by CTC clusters needs to be addressed to answer questions regarding their composition and role in the metastatic cascade, their interactions with stromal cells and eventually therapeutic implications. Currently it is known that CTC clusters may retain the expression of epithelial cytoskeletal and adhesion genes such as cytokeratin-14, E-cadherin, P-cadherin [53], or plakoglobin [45], but also strong expression of mesenchymal markers such as FN1 (fibronectin 1), CDH2 (N-cadherin), and SERPINE1/PAI1 (serpin peptidase inhibitor, clade E), FOXC1 (FN1 forkhead box protein C1) observed within the clusters [19]. The dynamics of epithelial-mesenchymal differentiation within the clusters is unknown. It has been postulated that mesenchymal transformation of epithelial cells mediated by TGF-β released from platelets may take place within the clusters [45], but the issue warrants further studies.

Staying Mesenchymal – Cooperation with Other Cells

The EMT process is induced in cancer cells by multiple cell types present in the tumor, like fibroblasts, mesenchymal stem cells, monocytes, or macrophages (reviewed in Ye and Weinberg [23]). Continuous secretion of EMT triggering signals allows for sustaining mesenchymal features in migrating cancer cells [55]. Even though EMT induction is being linked with hypoxia, an increased percentage of cancer cells with EMT phenotype can also be observed in vascularized areas of basal-like BC, what might represent the ability of endothelial cells to trigger EMT by secreting hepatocyte growth factor [56]. Thus,

Table 1. Clinical significance of CTC mesenchymal phenotype in breast cancer

Source	Type of cancer	Patients, *n*	Detection of CTCs				Detection of EMT markers in CTCs			
			blood volume, mL	enrichment method	CTC-positive patients, *n* (%)	assay for CTC detection/ profiling	epithelial markers	mesenchymal markers	patients with EMT-positive CTCs, *n* (%)	clinical relevance of EMT-positive CTCs
Aktas et al. [124], 2009	MBC	39	10	AdnaTest	69 (31)	RT-PCR	EpCAM MUC1 HER2	AKT2 PI3Kα TWIST1	43 (62)	Percentage of EMT+ CTCs higher in non-responders compared to responders
Raimondi et al. [126], 2011	BC	92	10	Anti-EpCAM Dynabeads	61 (66)	RT-PCR	CKs	Fibronectin VIM	31 (34)	ND
Armstrong et al. [136], 2011	MBC	16	15	CellSearch system	11 (69)	IF	CKs E-cadherin EpCAM	N-cadherin VIM O-cadherin	11 (100)	ND
Gradilone et al. [4], 2011	MBC	55	10	Anti-EpCAM Dynabeads	28 (56)	RT-PCR	CKs EpCAM	Fibronectin VIM	24 (48)	EMT+ CTCs correlated with shorter PFS
Kallergi et al. [129], 2011	BC	25 MBC 25 EBC	20	CD45 depletion	50 (100)	IF	CK19	TWIST1 VIM	25 (100) TWIST1+ MBC 25 (100) VIM+ MBC 16 (73) TWIST1+ EBC 17 (77) VIM+ EBC	Higher incidence of EMT+ CTCs in MBC compared to EBC
Markou et al. [127], 2011	BC	64 EBC 20 MBC	20	Anti-EpCAM Dynabeads	37 (58)	PCR-coupled liquid bead array	CK19	TWIST1	20 (31) EBC 4 (20) MBC	ND
Strati et al. [128], 2011	BC	66 EBC 26 MBC	20	Anti-EpCAM Dynabeads	48 (72)	RT-PCR	CK19	TWIST1	21 (42) EBC 10 (38.5) MBC	No difference in EMT+ CTCs between EBC and MBC
Barriere et al. [123], 2012	BC	61	7	AdnaTest	61 (100)	RT-PCR	EpCAM MUC1 HER2	AKT2 PI3Kα TWIST1	19 (31)	ND
Kasimir-Bauer et al. [125], 2012	BC	502	10	AdnaTest	97 (19)	RT-PCR	EpCAM MUC1 HER2	AKT2 PI3Kα TWIST1	129 (29)	EMT+ CTCs did not correlate with any clinicopathological factors
Mego et al. [152], 2012	EBC	52	5	CD45 and EpCAM depletion CellSearch AdnaTest	– 7 (18.4) 15 (35.7)	RT-PCR	–	TWIST1 SNAIL SLUG ZEB1 FOXC2	8 (15)	EMT+ CTCs not eliminated by neoadjuvant therapy
Mego et al. [131], 2012	MBC	21	7.5	CD45 depletion CellSearch	– 3 (14.3)	RT-qPCR	–	TWIST1 SNAIL SLUG	4 (21) SNAIL+ 5 (26) TWIST1+	High expression of EMT-TFs predictive of shorter PFS
Giordano et al. [185], 2012	HER2+ MBC	28	30	CD45 and CD326 depletion	9 (52.9)	RT-PCR	EpCAM CK19	TWIST1, SNAIL, ZEB1, and TG2	17 (60.7)	ND
Yu et al. [19], 2013	MBC	41	3	CTC-Chip	41 (100)	RNA-ISH	CKs EpCAM CDH1 (E-cadherin)	Fibronectin1 SERPINE1/PAI1 CDH2 (N-cadherin)	17 (41)	EMT+ CTCs associated with disease progression
Papadaki et al. [130], 2014	BC	80 EBC 50 MBC	10	Density gradient	13 (16) EBC 25 (50) MBC	IF	CKs	TWIST1	20 (80) MBC 4 (31) EBC	More EMT+ CTCs in MBC patients
Markiewicz et al. [20], 2014	EBC	117	5–10	CD45 depletion	54 (55)	RT-PCR	CK19 mamma-globin	VIM TWIST1 SNAIL SLUG	25 (26)	EMT+ CTCs associated with lymph node involvement

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Table 1 (continued)

Source	Type of cancer	Patients, *n*	Detection of CTCs				Detection of EMT markers in CTCs			
			blood volume, mL	enrichment method	CTC-positive patients, *n* (%)	assay for CTC detection/ profiling	epithelial markers	mesenchymal markers	patients with EMT-positive CTCs, *n* (%)	clinical relevance of EMT-positive CTCs
Markiewicz et al. [95], 2014	EBC	98	5–10	CD45 depletion	16 (17)	RT-PCR	CK19	VIM	12 (12)	All patients who died during follow-up were CTC+ (in the majority EMT+ CTCs)
Mego et al. [186], 2015	EBC	102	NS	CD45 depletion	25 (24.5)	RT-PCR	CK19	TWIST1 SNAIL SLUG ZEB1 FOXC2	13 (12.8)	ND
Polioudaki et al. [44], 2015	MBC	61	7.5	CellSearch	61 (100)	IF	CKs	VIM	27 (44.3)	Low CKs, considered as surrogate marker of EMT, correlated with tumor characteristics (TNBC and ER–) and shorter 1-year OS
Wang et al. [187], 2015	BC	221	7.5	No enrichment	48 (21.7) EpCAM+ 31 (14) CK19+	RT-PCR	CK19 EpCAM	VIM	39 (17.6)	No statically significant correlation between VIM and disease stage
Wu et al. [188], 2015	BC	18	5	Filter-based	12 (67)	RNA-ISH	EpCAM CKs	VIM TWIST1	7 (39)	EMT+ CTCs more frequent in MBC
Satelli et al. [151], 2015	MBC	58	7.5	CD45 depletion	48 (83)	Antibody-based separation	–	Cell surface vimentin (CSV)	48 (83)	EMT+ CTCs associated with progressive disease
Ueo et al. [42], 2015	BC	594	NS	No enrichment	389 (65.5)	RT-PCR	–	Plastin-3	389 (65.5)	Significantly poorer OS and DFS
Bulfoni et al. [142], 2016	MBC	56	7.5	CD45 depletion	8 (16)	Staining with antibodies (FACS); DEPArray	EpCAM E-cadherin	CD44 CD146 N-cadherin	37 (79)	Fraction of cells coexpressing epithelial and mesenchymal markers associated with poorer PFS and OS
Hensler et al. [189], 2016	BC	147	7	AdnaTest	4 (3.5)	RT-PCR	EpCAM MUC1 HER2	VIM	VIM significantly increased in CTCs fraction of patients ($n = 4$) compared to healthy controls ($n = 4$)	ND
Reijm et al. [155], 2016	MBC treated with aromatase inhibitors	45	7.5	CellSearch	ND	RT-qPCR	EpCAM	8 genes, including TWIST1	Higher expression of TWIST1 in poor responding group	8-gene CTC predictor discriminates good and poor outcome in first-line treatment with AI, CTC profile an independent predictor of PFS
Bredemeier et al. [156], 2016	MBC	62	10	AdnaTest EMT-2/ StemCellsSelect	ND	RT-qPCR	EpCAM EGFR HER2	9-gene panel including PI3Kα, AKT	56 (84) positive for at least 1 out 9 genes; PI3Kα, AKT expression below 10% of patients	ND

AI, aromatase inhibitors; CKs, cytokeratins; DFS, disease-free survival; EBC, early breast cancer; IF, immunofluorescence; MBC, metastatic breast cancer; ND, not determined; NS, not shown; OS, overall survival; PFS, progression-free survival; RNA-ISH, RNA in situ hybridization; RT-qPCR/RT-PCR, reverse transcription (quantitative) polymerase chain reaction; TNBC, triple negative breast cancer; VIM, vimentin.

endothelial cells of primary tumor vasculature would also count as stimulators of EMT in intravasating tumor cells. The underlying molecular mechanisms by which cancer cells outside of the primary tumor, like CTCs, maintain the EMT state have not been fully elucidated. Theories explaining the mesenchymal status of CTCs concern the role of interactions either with blood cells or with cancer cells within the clusters [57]. Platelets seem to be of special importance for metastasis, since thrombocytosis is frequently observed in patients with metastatic cancers [58]. Platelets facilitate invasion of cancer cells, intra- and extravasation of CTCs (by secreting lysophosphatidic acid) [59, 60], protect them from various host attacks, such as immune assaults [61, 62], apoptosis [63, 64] and shear stress [57, 65, 66]. Importantly, platelets may also contribute to EMT of CTCs and more efficient metastases formation [67]. Recently, mesenchymal CTCs isolated from BC patients were found clustered with platelets, and gene expression profiling of these CTCs was enriched in the TGF-β signaling pathway [19]. CTCs themselves can trigger so-called tumor cell-induced platelet aggregation, which activates platelets and leads to their attachment to the surface of CTCs by a GPIIb-IIIa-fibrinogen bridge and upregulated P-selectin. Tumor cell-induced platelet aggregation promotes platelets to release α-granules, which contain high levels of TGF-β and platelet-derived growth factor, both considered to be powerful activators of EMT [57]. Platelet-derived TGF-β and direct platelet-tumor cell contacts synergistically activate the TGF-β/SMAD and NF-κB pathways in cancer cells, resulting in their transition to an invasive mesenchymal-like phenotype and enhanced metastasis in vivo [67]. It requires further studies to clearly show whether platelets can induce EMT in epithelial cancer cells present in circulation or whether they merely sustain the mesenchymal phenotype which intravasating tumor cells had acquired in the primary tumor. It is possible that during their short transition in circulation (estimated CTC half-life 1–2.5 h [68]), CTCs maintain the phenotype with which they entered the blood stream, and only at the distant site can changes in EMT status be observed. Beerling et al. [69] showed that the percentage of E-cadherin low tumor cells is the same (60%) in circulation and in single cells which disseminated to the lungs (60%), but drops to 20% at the stage of 2-cell clusters and further to 0% at the stage of 3-cell clusters in the lungs. This progressive decrease in mesenchymal features at a distant site could represent the time frame required for mesenchymal cancer cells to revert back to the epithelial state in the absence of strong EMT cues present in the primary tumor or in the circulation.

A unique example of cells which are able to provide EMT triggering signals, mostly TGF-β [19, 70, 71] during the whole metastatic cascade, within and outside of the primary tumor, are cancer-associated fibroblasts (CAFs). CAFs are not only part of the primary tumor niche, as CTCs were observed in heterotypic clusters with CAFs [46, 72]. In the original study of Duda et al. performed on mice carrying Lewis lung carcinoma, cancer cells were shown to be shed from the primary tumor together with CAFs, what decreased their apoptosis rate and facilitated formation of lung metastases [72]. In the same study CAFs were also identified in metastases to the brain (which does not contain fibroblasts) of lung, breast, kidney, and endometrial cancer patients, indicating relevance of this mechanism in various cancers [72].

EMT in Dissemination

In animal models it has been shown that EMT-induced cancer cells disseminate even at the very early stages, preceding detection of invasive tumor [73, 74]. To investigate early tumor cell dissemination in vivo, Hüsemann et al. [74] used the MMTV-Her2 mouse mammary tumor model and found increased Twist1 expression in hyperplastic lesions during primary tumor development. Concomitantly, these mice presented increased disseminated cancer cells in bone marrow at this stage, suggesting that EMT may be involved in CTC/disseminated cancer cell seeding. Consistently with these findings, in a K-*ras*-driven mouse pancreatic tumor model, pancreatic CTCs were detected at the premalignant stage of tumor progression [73]. The majority of these CTCs presented a mesenchymal phenotype and expressed ZEB2, indicating activation of the EMT program [73].

Not accounting for the tumor stage development, but still showing the role of EMT activation in increased CTC seeding is the study of Tsai et al. [75], who examined the number of CTCs in a mouse model of squamous cell skin carcinoma in response to TWIST1 induction. TWIST1 expression dramatically increased the number of CTCs compared with control mice, and these CTCs presented an EMT phenotype with loss of E-cadherin and expression of vimentin [75]. Similarly, in the MDA-MB-468 breast tumor xenograft model, expression of SNAIL and SLUG increased the presence of vimentin-positive CTCs [76], and in a 4T1 mouse model of BC, ectopic expression of miR-200, which inhibits EMT, reduced the number of CTCs seeded from the primary tumors, but resulted in a higher metastatic tumor burden upon intravenous injec-

tion [77]. Quantification of this niche-dependent advantage of an individual epithelial/mesenchymal phenotype was performed in an orthotopic MMTV-PyMT-driven mouse model of BC, which showed that the ratio of epithelial to mesenchymal cancer cells is 100:1 in primary tumor, 15:1 in blood and 150:1 in lung metastases, presenting an advantage of the mesenchymal phenotype in circulation [22]. In another study of the MMTV-PyMT-driven BC mouse model [69], the disproportion between epithelial and mesenchymal CTCs was not so significant, however much more widespread. Epithelial CTCs constituted from 0 to 80% (average 40%) of CTCs detected. Despite this difference, both studies show enrichment of the mesenchymal phenotype of cancer cells in circulation. While trying to translate this discovery to the clinical situation, we have to keep in mind that MMTV-PyMT-driven BCs create tumors of the luminal subtype [78], thus not fully recapitulating the heterogeneity of subtypes seen in BC patients, which show differences in the percentage of the epithelial/mesenchymal phenotype of CTCs depending on the tumor subtype [19].

Despite the large amount of data supporting the role of EMT in tumor progression, experimental data do not show a uniform picture regarding metastatic competence of the epithelial and mesenchymal phenotype. Pioneering work of Tsui et al. [79] presented that tail vain, but not subcutaneous, injection of epithelial cancer cells is sufficient for the establishment of lung metastases, suggesting inability of epithelial cells to intravasate. On the other hand, neither subcutaneous nor tail vain injection of mesenchymal cells resulted in formation of lung metastases (despite the presence of mesenchymal CTCs in the circulation), which indicated inefficient colonization by mesenchymal cells. Only subcutaneous injection of a mixture of epithelial and mesenchymal cancer cells resulted in formation of lung metastases, which indicates cooperation between the two types of cells during the intravasation process [79]. In in vitro tests and in vivo mouse models, prostate cancer cells of the mesenchymal phenotype were also shown to increase the metastatic capacity of normally poorly motile, but proliferative epithelial cancer cells [80]. This cooperative model has recently been challenged, as Ras-transformed human mammary cells of mesenchymal phenotype, when implanted to mammary fat pads of NOD/SCID mice were shown to seed lung metastases as opposed to their epithelial counterparts [101]. In other models, inhibition of EMT did not influence the rate of lung metastases [22] or was similar between epithelial and mesenchymal phenotypes [69]. Trying to draw conclusions from these studies, we need to remember that model-dependent factors might account for the observed differences, as multiple factors – way of tumor cell injection (tail vain vs. mammary fat pad), presence of the immune system, cancer subtype and its inherent malignancy, marker used for defining EMT status as well as the ability of cancer cells to transit between the epithelial/mesenchymal state – can influence the results.

EMT and Stemness

In classical definition, stem cells (SCs) are self-renewing cells, which through life are able to recapitulate heterogeneity of the cell population in an organ in which they reside [81]; often they are also considered to be slow-dividing cells [82–86]. By analogy, cancer stem cells (CSCs) would be able to indefinitely recreate heterogeneity of the primary tumor [87, 88]. In cancer, numerous markers have been described to be expressed on cells with SC properties – to name the few most common in solid tumors: ALDH1, CD133, CD90, α_6-integrin, members of the ABC transporters, or combination of CD44/CD24 status [89]. Cancer cells of the mesenchymal phenotype were shown to be enriched in these stem cell markers [90–92], and induction of EMT in mutant HRAS-transformed immortalized human mammary epithelial cells and basal BC cell lines resulted in the generation of cells with the SC phenotype (CD44+CD24–/low) from non-SC (CD44low-CD24+), enriched in tumorigenic properties in vivo [90, 91, 93]. Although the mechanism is not universal to all BC cell lines (inefficient generation of cancer SC-like cells in luminal BC cell lines [93]), it clearly points to plasticity of the CSC-like state, allowing for easy transit between SC and non-SC phenotype. However, individual CSC markers do not detect a single population of cells with enriched tumorigenic properties. Studies on clinical samples of BC revealed that ALDH1-expressing, proliferative cells are present in the tumor center and exhibit an epithelial phenotype, whereas CD44+/CD24–, quiescent cells are located at the tumor periphery and are mesenchymal [94]. Interestingly, both CSC phenotypes were highly plastic, able to interconvert between both epithelial and mesenchymal CSC states. As the rate of proliferation is one of the factors determining sensitivity of cancer cells to chemotherapy and ability to colonize a distant site, proliferative nonmigratory epithelial cells would be more sensitive to chemotherapy but more capable of establishing metastases. Following this reasoning, mesenchymal cells would represent a transitory CSC-like phenotype allowing cancer cells to disseminate from primary

tumor and reach a distant site where a tumor growth is recapitulated by CSC-like cells which reverted back to epithelial state. Indeed, epithelial traits of metastases are often more pronounced than in the corresponding primary tumors [95–98]. Also our study in BC showed that loss of E-cadherin is observed in 37% of primary tumors but only in 2% of matched lymph node metastases [95]. Interestingly, albeit there is strong E-cadherin staining, metastases consistently showed a decreased rate of proliferation and increased expression of EMT transcription factors TWIST1, SNAIL, and SLUG in comparison to primary tumors. Expression of EMT transcription factors TWIST1 and/or SLUG correlated with cell division rate in primary tumors and lymph node metastases, with 50% reduction in the Ki-67 labeling index in TWIST1 and/or SLUG+ samples, underlining the inhibitory effect of EMT-TF on cell division. Identification of E-cadherin-positive, EMT-TF-expressing cells could also point to the presence of cancer cells with partial EMT states. Along this line, Del Pozo et al. [99] showed in a BC mouse model that metastasis-initiating cells (MICs, CD24+/CD90+) presented more mesenchymal features (vimentin, TWIST1, SNAIL, SLUG, ZEB1 expression, E-cadherin downregulation); however, heterogeneity of EMT markers was observed, indicating frequent partial EMT states. Cells with partial EMT phenotype were characterized by increased tyrosine kinase receptor ALX expression. Additionally, MICs were able to activate fibroblasts more efficiently than non-MICs, and their gene expression signature was related to extracellular matrix remodeling (matrix metalloproteinases, collagen and fibronectin expression), supporting their role in metastatic niche preparation [99]. Moreover, MICs secreted CCL2, which attracts monocytes, recently shown to be supportive of metastasis growth at the very early stages of metastasis initiation [100].

In a mouse model of BC, mesenchymal cancer cells when forced to revert to the epithelial state (MET) reduced the frequency of CSC phenotype (CD44+/CD24–), which was linked with increased sensitivity to a range of drugs: doxorubicin, paclitaxel, proteasome inhibitors and MAPK/EGFR inhibitors [101]. Also, in a BC model, upon treatment of mice with cyclophosphamide, cells which had undergone EMT were more resistant and abundant than epithelial cancer cells [22]; inhibition of EMT during concurrent chemotherapy administration resulted in reduced numbers of lung metastases [22]. This increased ability to sustain toxicity might be pertinent to cancer cells in the mesenchymal state, which were shown to have higher expression of multidrug resistance pump breast cancer resistance protein and P-glycoprotein as well as DNA-repairing enzyme O^6-methylguanine-DNA methyltransferase, H2Afx, Xrcc6 [92]. In a study of MBC [4], a statistically significant correlation was found between the expression of multidrug resistance proteins and ALDH1 in CTCs, as well as a shorter progression-free survival (PFS) time for patients whose CTCs expressed two or more multidrug resistance proteins.

EMT in Immune Escape

Melanoma is considered to be one of the most immunogenic cancers, with multiple melanosome differentiation markers triggering the immune response, like tyrosinase [102, 103], TRP2 [104] or gp100 [105]. Thus, melanoma is frequently used in immuno-oncology research to study how tumor and its microenvironment are shaped by the immune system [106]. Such interactions can be studied in detail in animals with a functional immune system; thus, syngeneic mouse models are frequently used. In one of such studies [107] performed in a C57BL/6 mouse model injected with the melanoma cell line HCmel3, downregulation of a large panel of melanoma differentiation markers was observed in relapse tumors growing in the inflammatory tumor microenvironment. Interestingly, increased expression of proinflammatory cytokines (IL-1β, TNF-α, IL-6, IFN-γ), enhanced by infiltration of CD8+ T cells and CD11b+ myeloid cells, occurred concomitantly with decreased recognition of cancer cells by T cells. Spindle shape morphology and gene expression profiling of cancer cells suggested their mesenchymal-like phenotype [107]. Similarly, a decrease in melan A expression in various melanoma cells following TGF-β incubation (and a switch to the mesenchymal phenotype) was linked to their decreased clearance by melan A recognizing CD8+ T cells in vitro [108]. In a syngeneic mouse BC model, wild-type FVB/N mice injected with neu-expressing (mouse version of HER2) tumor cells with epithelial morphology gave rise to neu-negative tumors showing mesenchymal morphology, consistent with induction of the EMT phenotype [92, 109]. In this model, CD4+ T cells were found to be primarily responsible for killing neu+ tumor cells, whereas CD8+ T cells were promoting EMT in cancer cells.

Tumors can evade the immune system not only by downregulation of highly immunogenic proteins, but also via reducing the exposure of their entire protein content (potential neoantigens) to immune cells. Lung cancer cells of mesenchymal phenotype show reduced

expression of immunoproteosome subunits (PSMB8, PSMB9, PSMB10) specializing in processing peptides to be presented on the cell surface and express less than half of their proteins on HLA type I molecules than epithelial lung cancer cells do, which allows them a more successful evasion of the immune system [110].

Moreover, repertoire of EMT-linked immune escape strategies is not restricted to hiding from the immune system, but covers also direct inhibition of immune cells. PD-L1 expression, which transmits a potent inhibitory signal to T cells, correlates with the EMT signature of BC (from microarray gene expression profiles) as well as loss of E-cadherin and acquisition of vimentin expression in immunohistochemically analyzed BC sections [111]. TGF-β treatment of basal/mesenchymal BC cell lines and primary luminal epithelial cells isolated from the breast can further upregulate PD-L1 expression on their surface [111]. Similarly, in primary tumors of colorectal cancer patients PD-L1 expression was found in cells with signs of EMT (E-cadherin downregulation or vimentin expression) [112]. Additionally, in vitro coculture of breast, lung, and liver cancer cell lines induced to undergo EMT with pro-inflammatory cytokines (TGF-β, IFN-γ, TNF-α) resulted in activation of various, tumor type-specific immunomodulatory functions on different subpopulations of immune cells, like reduction in NK cells and T-cell proliferation or induction of regulatory T and B cells [113]. In a melanoma mouse model in vivo as well as in human melanoma cells in vitro, SNAIL+ cancer cells of the mesenchymal phenotype trigger immune suppression by inducing regulatory T cells and impaired dendritic cells [114].

Tumor cells themselves, but also EMT-promoting stimuli, may assist immune escape. TGF-β, a known inducer of EMT, can promote an immunosuppressive environment within the tumor by directly influencing cells of both innate and adaptive immunity. TGF-β was shown to inhibit on multiple levels antigen-presenting cells, NK cells, and T cells as well as induce generation of regulatory T cells which support tumor growth [115, 116]. Previously mentioned platelets, by coating CTCs, can create a physical barrier protecting CTCs with downregulated MHC class I molecules from NK cell attack [117] or transfer MHC class I molecules to cancer cells allowing molecular mimicry [118]. The role of platelets in metastasis was in detail reviewed in Lou et al. [57] and Leblanc and Peyruchaud [119].

Although signs of immune evasion were mostly found on post-EMT cancer cells, not only the mesenchymal phenotype allows to evade the immune system. Gene expression profiling of EpCAM+ CTCs from colorectal patients showed increased (in comparison to primary tumor) expression of CD47, which acts as phagocytosis inhibitor [120]. And E-cadherin, as a ligand of inhibitory killer cell lectin-like receptor G1 (KLRG1) on NK cells, is able to suppress the function of NK cells by lowering their ability (via antibody-dependent cellular toxicity) to destroy HER2-overexpressing, trastuzumab-treated BC cells [121].

A graphical summary of the above described features of cancer cells linked to EMT and metastatic competence is presented in Figure 1.

Clinical Significance of EMT in CTCs

Prognostic Significance

Since the first report on CTCs in 1869 [122] numerous studies on the clinical significance of CTCs in different tumors have been published [5]. The prognostic value of CTC enumeration in BC [6–9], prostate [10], colorectal [11], and lung cancers [12], and many other cancers has been described. In MBC, CTCs count is a level-of-evidence 1 prognostic dynamic biomarker [13]. However, the actual utility of CTCs still remains largely academic. One of the reasons for the limited clinical use of CTCs is the inability to accurately distinguish highly aggressive from less aggressive cells. The characteristics of clinically relevant CTCs remain the key issue. EMT-associated changes of phenotype have been postulated to be responsible for the aggressiveness of CTCs.

To date, the majority of research studies reporting on the existence of EMT in CTCs has been performed in patients with BC (Table 1). Several groups have evaluated the expression of 3 EMT markers: AKT2, PI3Kα, and TWIST1; the studies have consistently found that about one third of BC patients have CTCs positive for at least 1 of these EMT markers [123–125]. In another study of BC patients, cells negative for CK/CD45 transcripts but positive for vimentin and fibronectin were detected in 34% of the patients [126]. Another 2 groups reported TWIST1-positive CTCs being detected in 31.2 and 38.5% of BC patients, respectively [127, 128]. Yu et al. [19] using 7 pooled epithelial transcripts (cytokeratins 5, 7, 8, 18, and 19; EpCAM and E-cadherin) and 3 mesenchymal transcripts (fibronectin, N-cadherin, and SERPINE1/PAI1) found that mesenchymal features of CTCs in MBC patients varied according to BC subtype. In patients with triple negative or HER2+ tumors, mesenchymal CTCs were the major subpopulation of CTCs. Interestingly, the authors also reported that expression of the mesenchymal

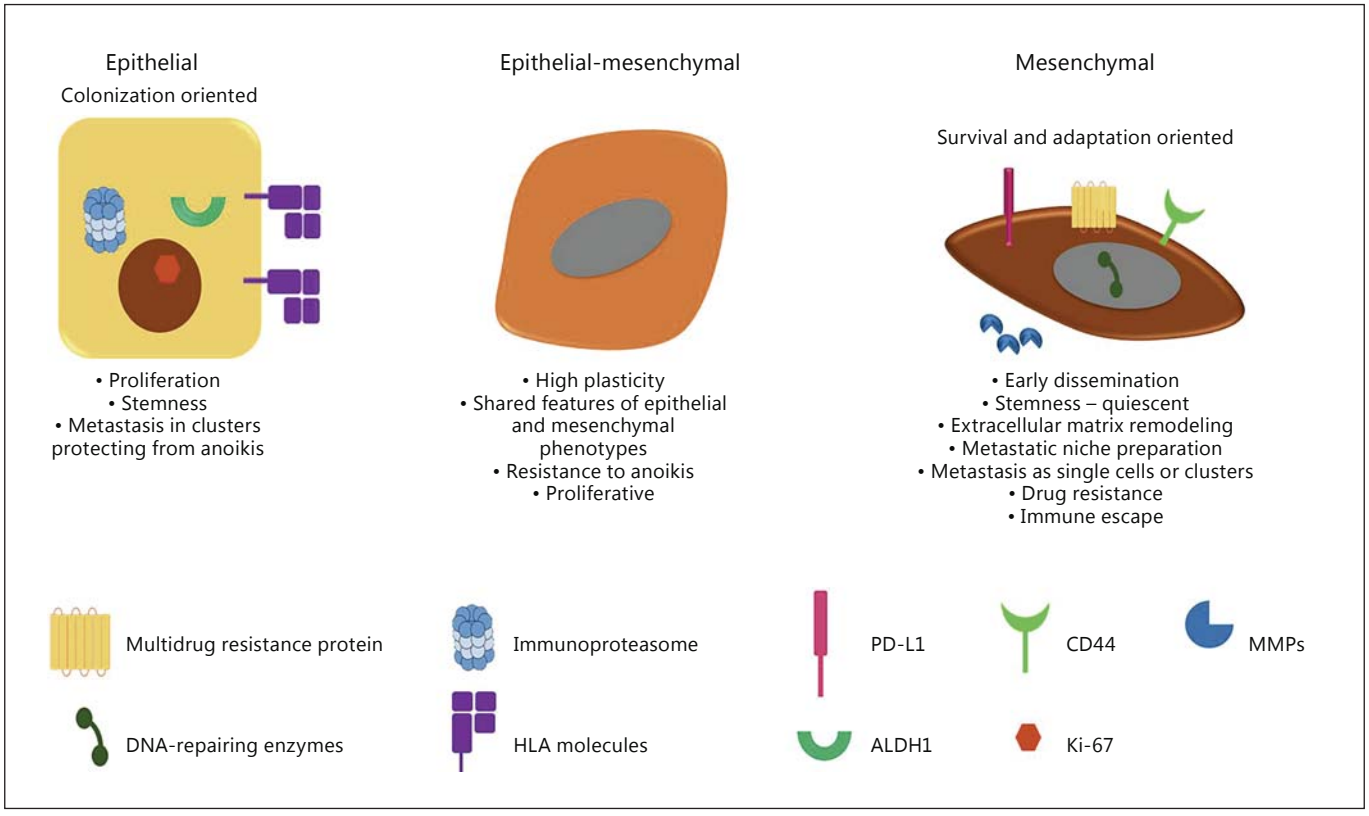

Fig. 1. EMT-induced changes in cancer cells. EMT can generate multiple phenotypes in cancer cells, linked not only to differences in expression of epithelial and mesenchymal markers, but also to polarity (apicobasal in epithelial cells and front-back polarity in mesenchymal cells), stemness, proliferative capabilities, as well as invasion and migration pattern (secretion of extracellular matrix-degrading metalloproteinases, MMPs, by mesenchymal cells). Whereas epithelial cells seem to be more capable of establishing tumor colonies at distant sites (proliferative phenotype – Ki-67+), mesenchymal cells are more resilient to changing conditions, thus are better equipped to survive during the metastatic cascade. Focusing on differences between cells with different EMT phenotypes, in mesenchymal cancer cells immunogenicity might be reduced due to a decrease in immunoproteasome levels, decrease in the number of HLA molecules presenting potentially antigenic protein on the cell surface or increased expression of immune cell-inhibitory molecules such as PD-L1, allowing mesenchymal cancer cells for more efficient immune escape. EMT was also shown to induce different stemness features to cancer cells – ALDH1 characterizes a subpopulation of cells with stem cell characteristics, which are proliferative, whereas the CD44+/CD24– phenotype is characteristic of quiescent mesenchymal stem cells. Additionally, mesenchymal cancer cells seem to be more resistant to therapies due to presence of multidrug resistance proteins, protecting cells from accumulation of toxic compounds. Additionally, expression of proteins involved in the repair of damaged DNA can be observed in mesenchymal cancer cells. Between phenotypes on opposite sides of the EMT spectrum, a range of intermediate phenotypes can develop. These cells characterized as having expression of both epithelial and mesenchymal markers, share features of both phenotypes, like resistance to anoikis (mesenchymal feature) or proliferative phenotype (epithelial feature). Exact characteristics of such cells will be dependent on the extent of EMT activation. Cells with a mixed epithelial-mesenchymal phenotype are thought to be highly plastic, thus very responsive to changes in the environment.

markers was more likely to be associated with clusters of CTCs rather than a single set of migratory cells [19].

Presence of mesenchymal markers was higher in MBC patients than in early stage BC patients [129, 130] and was associated with lymph node involvement [20], suggesting that EMT phenotype is directly related to the metastatic potential of CTCs. However, plastin-3 expressed in CTCs undergoing EMT was found with a similar frequency in all stages of BC, from I to IV [42].

Mesenchymal CTC detection correlated with poor patient outcome both in early [95] and metastatic BC [4]. Presence of mesenchymal CTCs was associated with shorter survival in MBC [4], which was also confirmed using a single CTC analysis approach [44]. MBC patients

undergoing high-dose chemotherapy followed by autologous hematopoietic stem cell transplantation with high expression of EMT-TF had shorter PFS compared to patients with low expression [131]. Furthermore, in the study of 594 BC patients of all stages mesenchymal CTCs were shown to be an independent prognostic marker of OS and disease-free survival in multivariate analysis [42]. Also, in early BC detection of mesenchymal CTCs correlated with patients' deaths [95]. Importantly, mesenchymal CTCs allowed a more accurate prediction of prognosis than the expression of epithelial markers alone [4].

Clinical significance of mesenchymal CTCs was examined not only in BC, but also in other cancers, including colorectal [43, 132], hepatocellular [133, 134], gastric [135], prostate [41, 136], bladder [137], ovarian [138], endometrial [139], and non-small cell lung cancer [140]. The majority of data concern the metastatic setting. In general, similarly to BC, mesenchymal CTCs seem to be associated with metastasis [43, 134] and poor patient outcome [43, 132]. Expression of EMT-related transcripts (PI3Kα, TWIST1 and AKT2) in CTCs from bladder cancer patients correlated with clinical stage and could be detected in patients negative for epithelial transcripts [137].

Currently, the focus is put on different states of EMT, with the emerging notion that partial EMT, but not necessarily a complete EMT, is associated with aggressive tumor progression. Armstrong et al. [136] examined the occurrence of EMT in CTCs from patients with progressive metastatic solid tumors, including castration-resistant prostate cancer and MBC, and showed that the majority (>80%) of CTCs coexpressed epithelial proteins along with mesenchymal proteins. CTCs from metastatic prostate cancer patients were found to exist in phenotypic states of epithelial-mesenchymal plasticity [141]. In patients with advanced non-small cell lung cancer, the majority of isolated or clustered CTCs harbor a dual epithelial/mesenchymal phenotype, and only some CTCs express exclusively vimentin or fibronectin [140]. Importantly, a fraction of cells coexpressing epithelial and mesenchymal markers was associated with poorer PFS and OS in MBC [142]. In lung cancer patients it was shown that radiotherapy can induce release of single CTCs or CTC clusters [143]. Comparative analysis of CTC numbers (as single cells or clusters) before and after irradiation showed an increase in CTC number (CK+/CD45–/DAPI+) in 50% (low radiation group) to 77% (high radiation group) of the patients [143].

Overall, presence of CTC clusters was associated with a significantly worse clinical outcome as compared with the presence of single CTCs [45, 54, 144]. In BC, CTCs were identified in 54 out of 79 patients (68%). Patients with detectable clustered CTCs had a significantly shorter PFS than with single CTCs [45]. Another prospective study confirmed the prognostic significance of CTC clusters in BC showing that the presence of CTC clusters was a strong independent predictor of PFS in advanced-stage BC patients, particularly in patients with inflammatory BC [144]. In prostate cancer, the presence of CTC clusters strongly correlated with a shorter OS [45]. Also in small cell lung cancer, presence of CTC clusters was an independent prognostic factor correlated with significantly shorter PFS and OS compared with the absence of CTC clusters [54].

Predictive Significance

Although increasing data and clinical trials show that CTCs can improve the prognostic accuracy, their predictive role is less extensively reported; particularly scarce are data concerning the predictive role of mesenchymal CTCs in this context.

The majority of reports on the possibility of monitoring therapeutic efficacy concern the enumeration of CTCs, based on EpCAM with the use of the CellSearch® system, which is the only system approved by the FDA up to date. When CTC detection in the context of neoadjuvant therapy is concerned, the GeparQuattro study (which is a large phase III trial, including the highest number of patients in this setting till now [$n = 213$]) reported no correlation between the persistence of CTCs and the treatment response of tumors [145]. This result remains in agreement with many other reports summarized by Bidard et al. [9], showing that CTCs detected by CellSearch® did not correlate with the response to therapy, measured by the completion of a pathological complete response. In contrast, a study conducted on 91 nonmetastatic patients with the Maintrack© cytology-based CTC detection system reported that different patterns of CTC decrease during adjuvant therapy were associated with complete response [146]. Studies conducted in the adjuvant setting reported a significant prognostic impact of CTC detection (with the CellSearch® technique). In the German multicenter SUCCESS study of more than 2,000 patients eligible for adjuvant chemotherapy, CTC positivity before chemotherapy was confirmed as an independent prognostic marker for disease-free survival and OS [7]. CTC positivity after chemotherapy had a marginally significant prognostic impact on survival. A single-center study conducted at the M.D. Anderson Cancer Center also reported that detection of CTCs predicted early recurrence and decreased OS in 304 chemonaïve patients with non-MBC [147]. A pooled anal-

ysis including studies in adjuvant and neoadjuvant settings reported CTCs (detected using CellSearch®) as an independent prognostic factor [8]. In metastatic disease the persistence of CTCs after treatment has been systematically shown to predict lack of responses to therapy [148–150]. CTC count superiority over serum tumor markers (CEA, CA15.3) was demonstrated in a pooled analysis of 1,944 patients. CTC count was associated with performance status, number of metastatic sites, elevated lactate dehydrogenase, elevated serum tumor markers, PFS, and OS. Moreover, adding CTC count and its change during therapy to an optimized clinicopathological model significantly increased the prognostic value of the model [9]. It was postulated that CTC count monitoring during therapy would allow early detection of resistance to therapy and ultimately improve the management of MBC patients.

The data concerning the predictive role of mesenchymal CTCs are very limited. No prospective clinical trials regarding the role of different phenotypes of CTCs were reported. Presence of mesenchymal CTCs was associated with progressive disease in both colon cancer and BC patients undergoing postsurgery adjuvant chemotherapy [38, 151]. MBC patients not responding to therapy showed increased levels of EMT markers compared to responders [124]. Moreover, primary BC patients treated with neoadjuvant therapy presented overexpression of EMT-inducing TF gene transcripts in their CTC fractions more frequently. As the number of complete responses in this subgroup was also lower, the authors concluded that neoadjuvant treatment was not able to eliminate CTCs with EMT phenotype [152].

Importantly, CTC phenotype changes during the course of treatment may serve as pharmacodynamic monitoring tools. In MBC patients not responding to treatment, the number of mesenchymal CTCs increased, while responders presented a decrease in CTC number and/or a proportional decrease in mesenchymal (compared to epithelial) CTCs [19]. These findings remain in agreement with studies highlighting the importance of EMT in conferring chemoresistance in BC and pancreatic cancer models [22, 153]. Recently, short-term expansion of CTCs in microwell-based culture was reported to predict response to anticancer therapy in early-stage, locally advanced and MBC [154]. Cluster formation in cell culture was affected by the presence and duration of systemic therapy, and its persistence may reflect therapeutic resistance as it correlated with shorter OS [154]. Also gene expression profiles in CTCs were reported to predict response to therapy with aromatase inhibitors [155] and during the course of palliative treatment in MBC [156].

In some cases estrogen receptor-α (ER) expression was shown to be downregulated in CTCs of BC patients, in comparison to their ER+ primary tumor [157, 158]. Heterogeneous or lack of expression of ER in CTCs of patients with ER+ primary tumor was linked with resistance to fulvestrant of BC patients [159]. Interestingly, downregulation of ER is associated with acquiring a mesenchymal phenotype of BC cells due to EMT [160–162]. One of the ER isoforms, ERα36, which might be expressed in ER-negative BC (lacking the classical ERα isoform of 66 kDa molecular mass) [163] was identified as being linked with the EMT phenotype of BC cells [164]. Also overexpression of HER2 in BC can result in a higher tumor burden, with cancer cells showing the EMT phenotype [165]. In a view of the EMT role in cancer, downregulation of ER and overexpression of HER2 can thus have a far more profound influence on the disease progression than just responsiveness to targeted therapy. Detailed studies are warranted to uncover the biological and clinical consequences of ER and HER2 expression in CTCs.

Targeting CTCs

Targeting Unique Cancer Markers

There are several ongoing large prospective interventional studies in which therapeutic decisions are based on CTC presence and/or CTC phenotypes in both non-MBC and MBC. The first prospectively randomized clinical trial that evaluated the clinical utility of CTCs was conducted by the SouthWest Oncology Group (NCT00382018). The trial showed no survival benefit for an early change of chemotherapy in patients with persistent or increased CTC counts following initial therapy [166]. The other trials focused on monitoring CTC count in MBC are ongoing. For example, the aim of the French CirCé01 trial (Circulating Tumor Cells to Guide Chemotherapy for Metastatic Breast Cancer; NCT01349842) is to evaluate repeatedly over several lines of chemotherapy whether patients whose CTC count does not decrease after the first treatment cycle benefit from a switch of chemotherapeutic regimens. In another French trial, the STIC CTC trial (NCT01710605), patients with hormone receptor-positive MBC are randomized between a standard clinician choice arm and a CTC count-driven arm.

Not only CTC count, but also CTC phenotype can be targeted. Preclinical and clinical data suggest that it is worth exploring the role of anti-HER2 strategies in women with HER2 nonamplified primary BC and detectable CTCs [167]. A single-center, randomized phase 2 study demonstrated the effectiveness of trastuzumab in eliminating CK19 mRNA-positive CTCs in BC patients after

prior chemotherapy exposure [168]. Furthermore the same group evaluated the efficacy of lapatinib, a dual EGFR and HER2 tyrosine kinase inhibitor, in therapy-resistant HER2-positive CTCs in MBC in a phase 2 single-center study (NCT00694252). Lapatinib was effective in decreasing HER2-positive CTC counts in patients with MBC, irrespectively of the HER2 status of the primary tumor [169]. Two prospective trials are currently ongoing to demonstrate the clinical benefit of anti-HER2 therapy in patients with MBC considered as HER2-negative and HER2-positive CTCs. The German DETECTIII study is a phase 3 study, comparing the lapatinib and chemotherapy combination versus chemotherapy alone in patients having at least 1 CTC with strong HER2 immunocytofluorescence (NCT01619111). A recent update on this study stated that about 19% of HER2-negative MBC patients were screened with at least 1 CTC with strong HER2 fluorescence [13]. The French CirCe T-DM1 (trastuzumab emtansine) study is a single-arm phase 2 study in which MBC patients with HER2-amplified CTCs receive T-DM1 as single therapy (NCT01975142). In this study, the discrepancy rate (patients with HER2-negative BC harboring HER2-positive CTCs) is lower than in the DETECTIII trial, around 5–8% [13].

Although there are several ongoing trials investigating the role of CTCs as a decision tool in MBC, there is only 1 trial on the clinical utility of CTCs in early BC, the EORTC Treat CTC trial. This trial is evaluating the efficacy of trastuzumab in patients with nonmetastatic HER2-negative disease and at least 1 CTC. The recent update of the trial showed that real-time screening of patients with early BC for CTCs, in the context of an international, multilaboratory trial, is feasible; first 26 patients were randomized [167].

The majority of described clinical trials are ongoing. The results of these trials will be crucial for evaluating the potential for CTC implementation in clinical BC management.

Recently a few preclinical studies reported the possibility of targeting EMT as the efficient way to inhibit metastasis. Suppression of MAPK7 kinase, responsible for maintaining mesenchymal cell properties in cancer cells, reduced the generation of CTCs and the appearance of lung metastases in a xenograft model of NOD scid gamma mice injected with the MDA-MB-231 BC cell line [170]. Forcing cancer cells in the mesenchymal state to revert to the epithelial one (MET) with forskolin or cholera toxin was shown to reduce the frequency of stem cell markers and increase their sensitivity to a range of drugs – doxorubicin, paclitaxel, proteasome inhibitors and MAPK/EGFR inhibitors [101]. Eribulin, a nontaxane microtubule dynamics inhibitor, induced a phenotypic switch from mesenchymal to epithelial states (MET), which was accompanied by decreased migration and invasiveness in vitro and decreased lung metastases and prolonged survival in an Mx-1 BC cell experimental metastasis model in vivo [171]. MET induced by eribulin was due to downregulation of the TGF-β/SMAD pathway [171].

Targeting Tumor-Specific Microenvironment

While relying on a surface receptor to target CTCs can be greatly challenged by cancer heterogeneity, targeting of tumor microenvironments has the advantage of recognizing a broader spectrum of cancer cells regardless of genetic differences or tumor types.

Since tumor cell interactions with host cells in the microenvironment also seem to be critical during CTC dissemination and travel through the circulation, many new strategies targeting these interactions are currently investigated. Targeting platelet-tumor cell interaction is one of the most promising strategies to reduce distant metastasis. One approach to minimize these interactions has focused on the use of anticoagulation agents. Agents such as recombinant mouse tissue factor pathway inhibitor [172] and the platelet aggregation inhibitor cilostazol [173] have been found to reduce the formation of secondary metastases. Several studies suggest that heparin, a powerful P-selectin inhibitor, can attenuate tumor metastasis in mice [174]. However, the use of anticoagulants may also adversely affect the normal hemostatic function of platelets in the case of bleeding. Therefore, a more focused approach aiming to block the signaling between platelets and CTCs is being developed. Invasive behavior can be induced by transforming growth factor-β_1 (TGF-β_1), which is secreted by activated platelets. Temporary contact with platelets is sufficient to induce invasive behavior in CTCs through TGF-β_1 [67]. Blockage of TGF-β_1 receptor I kinase activity through the use of SD-208, a small-molecule inhibitor, was shown to prevent the development of TGF-β-induced bone metastases in a melanoma mouse model [175]. Similarly, the use of another TGF-β_1 receptor inhibitor, IN-1130, suppressed EMT and the lung metastasis of mammary tumors in mouse models [176]. Accumulating data suggest that aspirin may act as an inhibitor of cancer metastasis, and the chemotherapeutic effects of aspirin on the metastatic process may depend on the inhibition of platelet function. In an analysis of 5 randomized trials in the UK on daily aspirin use at ≥75 mg, the risk of cancer with distant

metastases was also reduced [177]. Aspirin has been hypothesized to inhibit platelet-induced EMT of CTCs through the COX-1 signaling pathway, which may contribute to its antimetastatic effect [178]. Importantly, depletion of platelets or inhibition of TGF-β secretion solely in platelets drastically reduced distant metastases [67]. Another way to target CTC-platelet interactions could be targeted drug delivery to CTCs via platelet membrane-functionalized particles, as shown for a major tumor-killing cytokine, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), conjugated to the platelet membrane-coated silica particles which dramatically decreased lung metastases in a mouse BC metastasis model [179]. Taking into account the presented data, platelets are a promising therapeutic target for the attenuation of metastatic events.

Conclusion

As CTCs contribute to a large extent to metastasis formation, it is vitally important to characterize and treat them as accurately as possible, to increase the therapeutic efficiency and to reduce side effects. Exploring EMT processes also conveys chances for tumor therapy. EMT and the signal transduction pathways within this process might serve as potent targets for therapeutic approaches. Inhibiting these signal transduction cascades by specifically tailored drugs could help to diminish or even abolish metastasis formation, maybe even without doing harm to healthy cells. Analyzing and understanding the mechanisms which lead to EMT, especially in CTCs, which are the main root of remote metastasis formation, could therefore give rise to new treatment strategies. The relative rarity of CTCs in patients, especially at early disease stages, should also bring our attention to the representativeness of molecular characteristics of the few captured cells. Single-cell genomics [180–183] and transcriptomics [41, 138, 180, 182, 184] revealed significant interpatient molecular heterogeneity among CTCs.

We are beginning to explore the complex underlying mechanisms which lead from tumor cell dissociation from the primary tumor through EMT towards the formation of metastasis. Considering the described differences between epithelial and mesenchymal CTCs in therapy sensitivity, immune evasion, survival, and proliferative adventures, a detailed analysis of individual CTC phenotypes in cancer patients might offer promising perspectives for the future of personalized therapies.

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