2 Epithelial-Mesenchymal Plasticity in Circulating Tumor Cells, the Precursors of Metastasis

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Abstract

Circulating tumor cells offer an unprecedented window into the metastatic cascade, and to some extent can be considered as intermediates in the process of metastasis. They exhibit dynamic oscillations in epithelial to mesenchymal plasticity and provide important opportunities for prognosis, therapy response monitoring, and targeting of metastatic disease. In this manuscript, we review the involvement of epithelial-mesenchymal plasticity in the early steps of metastasis and what we have learned about its contribution to genomic instability and genetic diversity, tumor progression and therapeutic responses using cell culture, mouse models and circulating tumor cells enriched from patients.

Keywords

Breast cancer · Circulating tumor cells (CTCs) · Epithelial-mesenchymal plasticity (EMP) · Metastasis

2.1 Defining Epithelial-Mesenchymal Plasticity

Cancer metastasis, the major cause of patient mortality, is a complex multi-step process in which tumor cells become invasive, intravasate into the blood, survive in the circulation, extravasate out of the blood stream, and proliferate at the distal sites. During the early steps of metastasis, tumor cells lose apico-basal polarity through disruption of cell-cell interactions and cytoskeletal remodeling to support invasion [1]. These changes are reminiscent of the normal physiological process, epithelial to mesenchymal transition (EMT), that is required for gastrulation, neural crest cell migration, heart morphogenesis, organogenesis, and wound healing [1–7]. Utilization of EMT by cancer

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cells to migrate, invade, and survive when non-adherent provides an attractive model to understand the critical steps involved in the initiation of metastasis. The process of canonical EMT in cancer cells is generally attributed to epigenetic changes that are thought to be largely reversible upon removal of EMT stimuli, resulting in the reversion of this phenotype through mesenchymal-epithelial transition (MET). It is generally accepted that MET plays an important role in successful completion of the metastatic cascade with epithelial-like tumor formation [1, 7–11], and recently emerged controversies [12, 13] have been addressed [14, 15].

We will refer to these lineage switches as epithelial-mesenchymal plasticity (EMP) from this point forward to reflect the extensive bi-directional plasticity of the process. EMP phenotypes have been observed in cell culture and in mouse tumor models of breast, lung, prostate, pancreatic, colorectal, and ovarian cancers [16–18]. Detection of EMP in patient-derived tumor tissue specimens, however, has been complicated by the presence of stromal cells which express high levels of mesenchymal markers. As such, despite the dramatic invasive and tumorigenic phenotypes observed in mouse xenografts expressing EMP-regulating master transcriptional factors, Snail, Twist, and Slug among others, the direct observation of EMP in the metastasis of human epithelial cancers has remained elusive. Recently, tumor cells at various stages of EMP were detected in the blood of breast cancer patients, suggesting that EMP is not bimodal but is a continuous process [19]. These circulating tumor cells (CTCs) are extremely rare and are the putative precursors of metastasis. Therefore, defining EMP as a single dramatic transition between two states may be an oversimplification and may limit the study of EMP in some circumstances [7]. In this chapter, we will review the process of EMP, the evidence for EMP in clinical samples, its contribution to breast cancer dissemination (with a focus on the metastatic intermediates, CTCs), and the therapeutic implications associated with this process.

2.1.1 Epithelial-Mesenchymal Plasticity to Model the Early Steps of Metastasis

During EMP, epithelial cells within the primary tumor switch lineage to take on a more mesenchymal phenotype [1, 20], which is associated with morphological changes and molecular reprogramming [5, 21]. This consists of a series of sequential processes: the loss of apico-basal polarity due to cytoskeletal and junctional remodeling, increased cell migration as the result of decreased cell-cell adhesion and increased motility (sometimes at the cost of proliferation), and the acquisition of invasive properties such as passage through a basement membrane [1]. The basement membrane between the epithelia and nearby blood vessels is the first barrier encountered by invading cells [22]. Invasion requires breach of the basement membrane, then breakdown of the extracellular matrix in the stroma by proteases such as matrix metalloproteinases [23]. EMP regulates expression of many of the genes required for this breach of the basement membrane and matrix. Upon arrival at the secondary site, MET then proceeds in the reverse order, with increased polarity and cell-cell adhesion leading to decreased cell migration and an epithelial phenotype associated with increased proliferation. The steps of this process are highlighted in Fig. 2.1.

2.1.2 Inducers and Effectors of EMP

EMP in both development and cancer is induced and maintained by a variety of signals: (i) extracellular signals, (ii) master transcription factors, and (iii) post-transcriptional regulators. Extracellular signals regulating EMP consist of peptide growth factors (e.g. FGF, EGF, HGF, TGFβ), cytokines, differentiation factors (Wnt, Notch, SHH, NFκB pathways, RAS/receptor tyrosine kinases), and hormones secreted by the cancer cells themselves as well as the supporting cells in the tumor microenvironment [1, 7, 24–30]. Additionally, hypoxia and extracellular com-

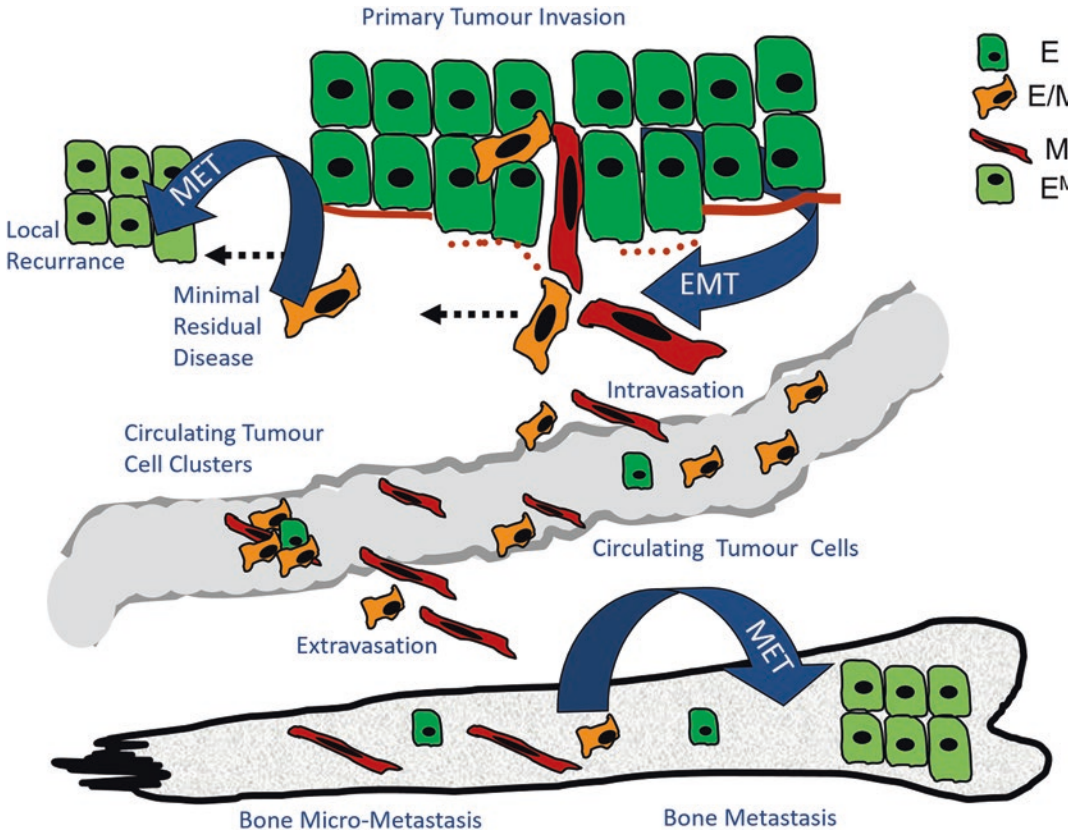

Fig. 2.1 Metastatic cascade highlighting CTC and EMP characteristics. A small proportion of carcinoma cells exhibit epithelial mesenchymal plasticity, resulting in hybrid (E/M) phenotype rather than a distinctly mesenchymal phenotypes (M). These mesenchymally shifted cells are associated with loss of the basement membrane and migration / invasion into the tumor microenvironment, where they can remain dormant. Epithelial change in these cells is likely to underpin local recurrence, allowing a new colony to form. A higher proportion of mesenchymally shifted (E/M) cells is found in the vasculature as circulating tumor cells (CTCs), indicating their increased capacity for intravasation and survival in the vasculature. A full spectrum of epithelial (E) to mesenchymal phenotypes is seen in the blood however, the hybrid phenotype dominates. CTC clusters containing cells at different stages of the EMP spectrum, and also normal immune cells and in some cases, tumor stromal cells, are also seen and have a higher prognostic value and a higher patho-biological potential. Dormant single cells / micrometastatic deposits can be seen in the bone marrow (depicted) or other metastatic sites. MET results in slightly altered gene expression profiles (E^M)

ponents such as collagen also can induce EMP [1, 25–29, 31–33]. These extracellular signals are transduced to transcription factors that regulate the expression changes required to elicit epithelial-mesenchymal state change. The master transcriptional regulators of EMP include Snail/Slug, Twist, and members of the Zeb transcription factor family [1, 34]. EMP is also regulated by post-transcriptional processes including ubiquitination, alternative splicing, and miRNAs that regulate protein translation, the most well characterized being the miR-200 family which modulates the expression of the (ZEB) proteins [1, 7, 34–36].

Along with a multitude of additional modulators and chromatin modifiers, these regulators coordinate the expression of proteins that maintain the epithelial state, apico-basal polarity, and

cell-cell adhesion, including Crumbs, PAR, Scribble, E-cadherin, α-catenin, γ-catenin/plakoglobin, and claudin. They also regulate proteins defining the mesenchymal state, cellular motility, and invasiveness, including N-cadherin, vimentin, and fibronectin [1, 7, 19, 34]. Together, these many inputs create a broad and often redundant signaling network to induce and maintain these states of plasticity in tumor cells [1, 2, 5, 25–27, 37–41].

2.1.3 EMP in Cancer Stem Cells and Drug Resistance

In addition to being involved in promoting metastasis, EMP has also been implicated in contributing to the maintenance of cancer stem cells (CSC). Like CSCs, cells undergoing EMP can survive under adverse conditions and exhibit resistance to chemotherapeutic interventions, although they do not necessarily self-renew [42]. Cells undergoing EMP coincidently acquire many CSC markers. In breast cancer, the presence of mesenchymal markers correlated with the presence of CSC markers including ALDH1, NANOG, OCT-4, and CD44 [43]. Double knockdown of the cancer stemness markers NANOG and OCT-4 reversed EMT in lung adenocarcinoma, while induction of these genes promoted EMT in breast cancer [43]. Similarly, knockdown of the OVOL2 transcription factor in nasopharyngeal carcinoma cells decreased both EMT and stemness [44]. Upregulation of CSC markers and the appearance of a CSC phenotype during EMP has been observed in cell lines, mouse models, and patient samples [43, 45–48]. However, EMP is not always associated with the appearance of CSC-like properties. CSCs consist of both mesenchymal and epithelial phenotypes under different contexts, while EMT is often associated with a more mesenchymal state [49]. Further, CSCs represent a minor population of all tumor cells, whereas EMP occurs in a much larger fraction of tumor cells suggesting that additional criteria are involved in defining the functional characteristics of CSCs [50]. Further studies are required to better define the relationship between EMP and CSCs, specifically whether they represent a common phenomenon and if they are both induced and maintained through the same inducers and pathways.

Across several cancer types, the mesenchymal state is associated with increased drug resistance while the epithelial state is associated with increased sensitivity [34, 51–53]. In a mouse model of breast cancer, cells forced to revert to the epithelial state lost CSC markers and were increasingly sensitive to doxorubicin, paclitaxel, proteasome inhibitors, and MAPK/EGFR inhibitors [54, 55]. Further, neoadjuvant chemotherapy in breast cancer has been shown to be ineffective against CTCs in the EMP state [56, 57]. EMP signatures were also found to be associated with treatment response and resistance in non-small cell lung carcinoma, pancreatic, breast, and ovarian cancer [5, 12, 13, 30, 58–60]. The mechanistic aspects of EMP thought to confer drug resistance are similar to those in CSCs and include elevated expression of antiapoptotic proteins and drug efflux transporters and immunosuppression through the activities of EMP master transcription factors [50, 61].

2.1.4 Significance of EMP in Non-epithelial Cancers

While EMP is important for tumors of epithelial origin to migrate to the metastatic site, tumors of non-epithelial origin – leukemias, lymphomas, myelomas, sarcomas, and brain and spinal cord cancers – do not necessarily encounter these barriers. For some non-epithelial cancers, such as glioblastoma, markers of EMP are still induced by microglia and macrophages via NFkB and support invasiveness [30, 62, 63]. Further, of the four glioblastoma subtypes, the mesenchymal subtype is the most aggressive and radioresistant [64–66]. In sarcomas such as osteosarcoma and

rhabdomyosarcoma, where the cell of origin is already highly mesenchymal, further upregulation of the EMP transcription factor ZEB1 has been observed compared to normal tissue, and SNAIL expression was associated with poorer overall survival [67–69]. Higher expression of epithelial E-cadherin is also associated with improved survival in bone and soft tissue sarcomas [70].

2.1.5 Contribution of EMP to Genomic Instability and Genetic Diversity

A series of studies published several years ago showed that mitosis during Drosophila and Xenopus embryogenesis is actively inhibited in cells undergoing gastrulation. Premature induction of proliferation before the completion of gastrulation in cells undergoing EMP results in extensive developmental abnormalities [71–74]. Recent studies show that this embryonic process is exploited by the tumor cells to drive genomic instability and diversity [75] – changes that can have profound consequences on tumor progression and drug responses. Although transitioning of epithelial cells to a mesenchymal state is reversible upon removal of the EMP inducers, the induced abnormalities in ploidy and genomic heterogeneity are heritable. The mechanistic clue to this incompatibility came from detailed proteomic analysis, which revealed that several nuclear envelope proteins are suppressed as epithelial cells transition to a mesenchymal state. Nuclear envelope proteins, in addition to providing the structural framework of the nucleus and selectively modulating the passage of molecules between the cytoplasm and the nucleoplasm, also play critical roles in orchestrating proper mitosis. Therefore, while the suppression of nuclear envelope proteins reduces the rigidity of the nucleus to facilitate EMP-associated migration and invasion, the requirement of these proteins for mitosis [76, 77] might also render their decrease during EMP incompatible with simultaneous proliferation. Subsequent studies show that clonal epithelial populations spontaneously generate mesenchymal variants, which can revert to an epithelial phenotype [78] contributing to chromosome instability and the selection of robust variants capable of forming metastatic tumors. Disruption of tissue architecture associated with this cell fate switch has also been implicated in maintaining the fidelity of chromosome segregation [79].

2.1.6 Mouse Models of EMP

The inherent plasticity of EMP makes unequivocal determination of the lineage for a given cell difficult. Most studies evaluating the role of EMP in disease progression in vivo have relied on xenograft mouse models and cultured cells. Experimental induction of EMP in cancer cells led to an increase in metastasis, and knockdown of EMP or premature induction of MET reduced metastasis [47, 80–82]. Interestingly, expression of the EMT-inducing homeobox transcription factor, Prrx1, led to EMT phenotypes in cultured cancer cells [47]. However, loss of Prrx1 in cultured cells was required for efficient metastasis upon tail vein injection or orthotopic tumor formation in mice [47]. Other studies utilized mouse models with intrinsic EMP reporters and gain-of-function or loss-of-function of EMP master transcription factors [13, 20]. In skin-specific Twist1-inducible mice, Twist1 induction caused higher rates of squamous cell cancer development upon treatment with a carcinogen [83]. Reversal of this Twist1 induction upon tumor cell dissemination significantly increased metastasis. Together with the Prrx1 data, this result strongly supports a role for MET in metastatic outgrowth [83]. Single-cell lineage tracing with reporter genes irreversibly activated by lineage-specific promoters have been used to query the fate of the cells experiencing EMP. Reporter genes thus activated by epithelial/mesenchymal promoters have been used to track EMP in mouse models and monitor the change in cellular states during

the course of metastasis and tumor progression [84]. Breast cancer models have found that a small fraction of primary and metastatic tumor cells undergo EMT [12]. Conversely, pancreatic cancer models showed about half of tumor cells had undergone EMT, rarely occurring in premalignant lesions [85]. However, given the complex signaling networks involved in promoting EMP, it is difficult to reach concrete conclusions based on studies that rely on a single marker in a given model, particularly in the context of the EMP hybrid phenotype, where the degree of induction may be less strong.

2.1.7 Detecting EMP in Clinical Samples

Although lineage tracing in humans is not possible and acquisition of serial samples is quite difficult, evaluation of epithelial and mesenchymal markers in patient-derived tissue provides a snapshot of EMP in the clinical setting. Immunohistochemistry (IHC) of human breast cancer samples with mesenchymal markers such as vimentin, N-cadherin, cell cycle, and tumor specific markers such as HER2, showed evidence for EMT in triple negative and basal-like tumors but not in invasive lobular carcinomas [86, 87]. RNA *in situ* hybridization (RNA-ISH) using multiple probes to detect both epithelial and mesenchymal transcripts in the same samples delineated the ratios of epithelial and mesenchymal tumor cell populations at the single cell level in the primary tumors and draining lymph nodes of human breast cancer specimens [19]. While most tumor cells exhibited an epithelial phenotype, triple negative breast cancer was enriched for cells with mesenchymal markers, and all subtypes contained rare cells with combined epithelial and mesenchymal staining [19]. RNA-ISH analysis was also performed on CTCs from breast cancer patients, where it performed significantly better at detecting mesenchymal cells compared to standard cytokeratin approaches (discussed in more detail below) [19]. CTCs provide a non-invasive tool to monitor EMP in real time as patients progress through therapeutic interventions.

2.2 EMP in Circulating Tumor Cells

During metastasis, CTCs – the putative metastatic precursors – travel through the blood. Although the majority of CTCs are destroyed in the blood through apoptosis, the remaining viable cells reach and reside within distal sites in a dormant state until they adjust to the new microenvironment and eventually proliferate. The relative accessibility of CTCs in the peripheral blood provides real time sampling of tumor cells to interrogate the contribution of EMP to metastasis and drug responsiveness [30].

Studies of CTCs provide some of the best evidence for the involvement of EMP in promoting metastasis. Mesenchymal markers have been observed in CTCs from patients with glioblastoma, breast, liver, nasopharyngeal, colon, gastric, bladder, pancreatic, and non-small cell lung cancers [7, 19, 30, 54, 56, 80, 88–97]. A summary of EMP studies in breast cancer CTCs is shown in Table 2.1. These studies showed that CTCs are a heterogeneous population and, as predicted, exhibit more mesenchymal characteristics compared with the cells in the primary or metastatic tumors. A large fraction of individual CTCs was also found to express both epithelial and mesenchymal markers, suggesting that plasticity is a common component of the metastatic phenotype [7, 19, 30, 56, 80, 91–96]. Lineage tracing experiments in animal models will be required to explore the stage of tissue residence or circulation at which CTCs undergo both EMT and MET [7].

2.2.1 Hybrid-EMP and CTC Clusters

Recent studies regarding EMP in CTCs address a longstanding dispute in the field: whether EMP should be defined as a binary process with epithelial and mesenchymal endpoints as observed in most non-disease cases (with notable exceptions

Table 2.1 Detection of EMT in CTCs from breast cancer patients

EMT/CSC markers (+ other markers co-analyzed)	Nr of patients/ Breast cancer type	Method of CTC enrichment	CTC Detection/ characterization method	Identification/ epithelial markers	Correlation between EMT markers and clinical parameters (and other factors)	References
Twist1, PI3Kα, Akt2, ALDH1	39 M_1	AdnaTest BreastCancer	RT PCR	EpCAM, MUC1, HER2	CTC^{EMT+} higher in therapy non–responders	[98]
Vim, E-cad, N-cad, O-cad, CD133	16 M1	CellSearch EpCAM-isolation	IF	CKs	CTC^{EMT+} detected	[99]
Vim, FN	55 M_1	Epcam positive CELLection beads	RT PCR	CKs	CTC^{EMT+} patients↓ PFS	[100]
Vim, Twist1	25 M_0 25 M_1	Ficoll density gradient/ CD45 negative Dynal CELLection beads	IF	CKs	CTC^{EMT+} and EMT+ Circulating Tumor Emboli more numerous in MBC patients	[101]
Twist	64 M0 20 M1	EpCAM positive CELLection beads	PCR-liquid bead array hybridization	CK19 MGB1 HER2	EMT markers detected both in EBC and MBC patients	[102]
Vim, FN, ALDH1	92 M_0+M_1	EpCAM positive CELLection Dynabeads	RT PCR	CKs	CTC^{EMT+} correlation with disease stage	[103]
Twist	66 M_0 26 M_1	EpCAM positive CELLection beads	RT-PCR	CK19 MGB1 HER2	EMT marker detected both in EBC and MBC patients	[104]
Twist1, PI3K-α, Akt2, ALDH1, Bmi1, CD44	61 M_0	AdnaTest EMT-1/Stem Cell	RT PCR	-	dedifferentiated CTC (ddCTC) correlate with negative lymph node status	[105]
Twist1, PI3K-α, Akt2, ALDH1	130 M_0	AdnaTest Breast Cancer	RT PCR	EpCAM MUC1 HER2	No correlation found	[106]
Twist1, Snail, Zeb1, TG2	28 M_1	CD45/CD326 magnetic beads depletion	RT PCR	CKs	CTC^{EMT+} detected	[107]
Twist1, PI3K-α, Akt2, ALDH1	502 M_0	AdnaTest Breast Cancer	RT PCR	EpCAM MUC1 HER2	No correlation found	[108]
Twist1, Snail, Slug	21 M_1	CD34 depletion/ Ficoll density gradient/CD45 magnetic beads depletion	RT PCR	-	↓ PFS/early relapse in patients with high levels of EMT transcription factors	[109]

(continued)

Table 2.1 (continued)

EMT/CSC markers (+ other markers co-analyzed)	Nr of patients/ Breast cancer type	Method of CTC enrichment	CTC Detection/ characterization method	Identification/ epithelial markers	Correlation between EMT markers and clinical parameters (and other factors)	References
Twist1, Snail, Slug, Zeb1, FoxC2	52 M_0	CellSearch, AdnaTest Breast Cancer Ficoll density gradient + CD45 magnetic bead depletion	RT PCR	-	CTC^{EMT+} resist neoadjuvant therapies	[110]
CD44, CD47, MET	4 M_1	CellSearch RosetteSep hematopoietic cells depletion/ FACS EpCAM-CD45	FACS	EpCAM	$CTCs^{CD44+MET+CD47+}$ increase with disease progression and correlate with patient metastatic burden and ↓overall survival	[111]
N-cad,FN,PAI1,E-cad-	41 M_1	Herringbone–chip EpCAM, HER2, EGFR positive selection	RNA-ISH	CKs, EpCAM,	CTC^{M+} associate with disease progression CTC^{M+} observed as both single cells and multicellular clusters Reversible shifts between CTC^{E+}/CTC^{M+} accompanied each cycle of response to therapy.	[19]
Twist1, Snail, Slug, Zeb1	M_0	RosetteSep selection kit CD45 negative selection	RT PCR	CK19	CTC^{EMT+} associate with high tumor grade and expression of MMP-1 in tumors.	[112]
Vim, Snail, Twist1, Slug, uPAR	117 M_0	Density gradient centrifugation/ CD45 magnetic particle depletion	RT PCR	CK19 MGB1 HER2	CTC^{EMT+} associate with Lymph node involvement	[113]
Vim	98 M_0	Density gradient centrifugation/ CD45 magnetic particle depletion	RT-PCR	CK19 MGB1 HER2	CTC^{EMT+}predominantly detected in patients who died during follow up. CTC^{EMT+}more frequent in patients with low E-cad in the primary tumor.	[114]
Twist1, ALDH1	80 M_0 50 M_1	Ficoll density gradient+cytospins	IF	CKs	$CTC^{ALDH1high/NuclearTwist}$ more frequently detected in MBC	[115]
Vim, Slug, EGFR	78 M_0	Carcinoma Cell Enrichment and Detection kit, MACS technology CK magnetic beads positive selection	IF	CKs	CTC^{EMT+} correlate with tumor size	[116]
Ratio vim/CKs	61 M_1	CellSearch	IF	CKs	$CTC^{CK\ low}$ correlate with TNBC and shorter OS	[117]

Surface vimentin Twist1, Snail, FoxC2, E-cad	58 M_1	EasySep™ Human CD45 Depletion Kit + positive collection with surface vimentin ab magnetic beads	IF	EpCAM	CTC^{EMT+} count higher in treatment non-responders (association with disease progression)	[118]
Plastin 3	594 M_0+M_1	FICOLL density gradient	RT-PCR	–	Higher Pls3 levels in TNBC Pls3-positive patients have poorer OS/DFS	[119]
Vim	221 M_0+M_1	–	RT-PCR	EpCAM CK19 HER2	CTC^{EMT+} detected in all stages CTC count decrease after treatment	[120]
Vim, Twist1	18 M_0+M_1 (other type of tissues analyzed)	CD45 depletion/ CanPatrol (filtration system)/IF-FISH CD45	RNA-IF	EpCAM, CK8/18/19	CTC^{EMT+} higher in MBC patients	[94]
Vim, Tissue Factor	22 M_1	ScreenCell filtration device	IF	CKs	Detection of Vim+/Tissue Factor+ CTCs	[121]
N-Cad, CD44, CD146, E-cad$^-$	47 M_1	CD45 MicroBeads depletion/ DEPArray sorting CD45 negative cells	IF	EpCAM	CTC $^{EMT+}$ associate with shorter PFS/OS	[122]
Vim (55 breast cancer associated genes)	147 M_1	AdnaTest BreastCancer	RT PCR	EpCAM MUC1 HER2	Vimentin increased in CTC-enriched samples	[123]
EpCAM	32 M_1	Parallel multi-orifice flow fractionation chip (p-MOFF system)/ IF CD45-CK	IF	EpCAM, CK 7, 8	ND	[124]
Twist1	45 M_1 (before and after aromatase inhibitors)	CellSearch Profile Kit (EpCAM-positve Magnetic beads selection)	RT-PCR	CK8 within the 8 gene signature	96 genes analyzed, 8 gene-signature (including Twist1) for non-responders	[125]
Vim	22 M1 (before and after eribulin treatment)	microfluidic chip	IF	CKs	CTC^{EMT+} more frequently observed in TNBC Patients with more total CTCs and more CTC^{EMT+} have a shorter PFS Total CTC count increase with disease progression	[126]

(continued)

Table 2.1 (continued)

EMT/CSC markers (+ other markers co-analyzed)	Nr of patients/ Breast cancer type	Method of CTC enrichment	CTC Detection/ characterization method	Identification/ epithelial markers	Correlation between EMT markers and clinical parameters (and other factors)	References
Vim, Twist1	108 M_1	CanPatrol CTC filtration system	RNA-ISH	EpCAM CK8/18/19	PFS↓ in patients with CTC^{EMT+} Total CTC ↓ during chemotherapy but proportion of CTC^{EMT+}↑	[127]
E-cad, N-cad, Vim, Pls3, CD44, NANOG, ALDH1, OCT-4, CD133, plakoglobin	83 M_0	Density gradient centrifugation/ CD45 magnetic particle depletion	RT-PCR	CK19, MGB1, HER2	Different EMT CTC status defined in the cohort. Mesenchymal CTCs and epithelial-mesenchymal CTCs associate with lymph node involvement and larger tumor size. Mesenchymal CTCs correlate higher risk of death.	[128]

Vim vimentin, *FN* fibronectin, *CKs* cytokeratins, *Cad* cadherin, *MGB1* mammaglobin A, *Pls3* plastin3, *Ab* antibody, M_0 non metastatic breast cancers, M_1 metastatic breast cancers, *IF* immunofluorescence, *ISH* in situ hybridization, *OS* overall survival, *PFS* progression-free survival, *RFS* relapse free survival, *DFS* disease-free survival

of cohort migration as outlined below), or whether EMP is a spectrum phenotype with potentially stable higher-plasticity manifestations along the continuum from epithelial to mesenchymal phenotype [1, 53]. Highly-plastic cells with both epithelial and mesenchymal phenotypes (hereafter referred to as hybrid-EMP) are observed in many CTCs as well as in vivo; in fact, hybrid-EMP CTCs are more commonly observed than fully mesenchymal cells in many studies [8, 129]. Two recent studies of mouse pancreatic ductal adenocarcinoma (PDAC) and skin squamous cell cancer showed that these hybrid-EMP cells are more plastic than either epithelial or mesenchymal cells, with a higher ability to interconvert among the types in culture [8, 129]. Epigenetic, transcriptional, and post-transcriptional mechanisms were identified as regulating this interconversion, suggesting further study is needed to devise a unified mechanism for hybrid-EMP plasticity or to identify disease-specific mechanisms [8, 129]. Based on this data, we and others define EMP as a spectrum of phenotypes with highly plastic interconversion among the different states, with increased appreciation that the location of any particular cell along this continuum has important implications for both cancer and development [1, 7, 8, 129–137]. However, it is important to note that while the existence and importance of hybrid-EMP is accepted, it is still unclear whether it represents an intermediate phase during EMP or a final state, and even whether the same signaling pathways at work during EMP are also responsible for hybrid-EMP [7, 8].

Practically, the label hybrid-EMP is assigned to varied states, which include cells that downregulate epithelial markers but do not upregulate the full complement of mesenchymal markers, as well as cells expressing both epithelial and mesenchymal markers [8, 129]. For example, cells with upregulation of mesenchymal processes such as loss of polarity and increased motility and invasion but without loss of cell-cell adhesion or cell individualization. Indeed, although individual cell migration is a hallmark of EMT, recent studies have highlighted the presence of multicellular CTC clusters (up to 100 cells) in the circulation of patients with advanced cancers such as inflammatory breast cancer, and cohort migration is accepted as a frequent mode of invasion [1, 51, 138–147]. Clusters are more effective at colonizing secondary sites than single CTCs and correlate with a worse prognosis [7, 19, 80, 139]. Importantly, there is an association between CTC expression of mesenchymal markers and cluster formation. Many clusters are coated in platelets, which are a source of TGFβ and may help induce or maintain mesenchymal characteristics [30, 148]. These clusters necessarily maintain cell-cell contacts and some epithelial-like expression (notably the desmosomal protein plakoglobin), suggesting that they exhibit the hybrid-EMP phenotype described above [8, 51, 80, 149, 150]. Indeed, tumor spheres of hybrid-EMP mouse prostate cancer cells exhibited collective cell migration and cluster delamination while fully mesenchymal spheres only showed single-cell invasion [8]. It is not clear whether hybrid-EMP clusters are composed of a homogenous population of hybrid-EMP CTCs versus a mixed population of epithelial CTCs and mesenchymal CTCs [80]. However it is important to note that lineage tracing and tumor transplantation experiments show that CTC clusters do not form in the bloodstream through aggregation of single CTCs, but originate from polyclonal primary tumors, suggesting that the hybrid-EMP phenotype is established before invasion into the circulation [139, 151]. Although these clusters may seem impossibly large for invasion or extravasation through the blood vessel into the secondary tissue, studies have shown that CTC clusters can traverse the capillaries of Zebrafish by rapid reorganization into single-file chains [140]. Finally, although cohort migration is highlighted in the study of cancer CTCs, it should be noted that similar modes of invasion occur during development, wound healing, and mammary reorganization in some species, underscoring the fact that this hybrid-EMP phenotype is not restricted to the cancer environment [1].

2.2.2 Role of EMP in CTCs During Progression and Therapeutic Response

The implications of hybrid-EMP phenotypes on tumor histology and prognosis are significant. Hybrid-EMP cells are detected in both primary and metastatic tumors and are particularly prevalent in individual and clustered CTC populations as noted above [19]. Single-cell evaluation of both EMP markers and tumor-specific markers (such as HER2) in breast cancer confirm that these hybrid-EMP cells are tumor-derived [19]. Mesenchymal mouse PDAC tumors were poorly differentiated while hybrid-EMP tumors were moderately to well-differentiated [8]. Similar results are observed in human poorly differentiated quasi-mesenchymal, squamous, or basal-like PDAC tumors versus well-differentiated classical/exocrine-like, classical, or pancreatic progenitor/ADEX tumors [8, 152–154]. The proportion of breast cancer CTCs with fully epithelial, predominantly mesenchymal, or hybrid-EMP seems to be dependent on tumor type and stage, consistent with data for primary and metastatic tumor cells. Pre-invasive ductal carcinoma in situ (DCIS) lesions exhibit exclusively epithelial phenotypes, while invasive breast cancers contain rare hybrid-EMP cells, suggesting incomplete MET [19]. Further, CTCs from patients with lobular type (ER+/PR+) cancers were predominantly epithelial while CTCs from patients with HER2+ or triple negative breast cancers were predominantly mesenchymal [19].

Studies show that EMP phenotypes in CTCs indicate poor prognosis and resistance to therapy. Hybrid-EMP mouse skin cancer cells produced more metastasis after tail vein injection than fully mesenchymal cells [129]. In humans, EMP CTCs confer poor prognosis in breast, prostate, liver, colorectal, head and neck, pancreatic, endometrial, and lung cancers [155]. Hybrid-EMP cells are more anoikis-resistant and drug-resistant [53, 156], giving them a better chance of metastatic colonization. Although the signaling pathways mediating anoikis resistance are not fully understood, EMP markers such as TGFβ, Twist, Snail, and miR200 have also been shown to have effects on survival in circulation [157, 158]. This is particularly significant to breast cancer treatment, as apoptosis is the main inducer of regression in systemic therapy and resistance of disseminated tumor cells to apoptosis is correlated with worse prognosis [159]. EMP CTCs were also associated with chemotherapy or radiotherapy resistance in ovarian, breast, and colorectal cancer [109, 160, 161]. Interestingly, when one breast cancer patient was followed longitudinally, mesenchymal-CTCs decreased with therapy response and then increased upon development of resistance, a phenomenon that was observed over two successive rounds of treatment. This increase in mesenchymal-CTCs was accompanied by the appearance of CTC-clusters [19].

2.2.3 The Influence of EMP on CTC Isolation Technologies

The plasticity of mixed epithelial and mesenchymal CTC phenotypes in the blood has been highly consequential in defining the capture efficiency of antibody-based CTC isolation approaches that rely on the expression of the epithelial marker EpCAM on the surface of tumor cells. The only FDA approved technique for in vitro diagnostic use, CellSearch®, (Veridex, Menarini Silicon Biosystems) uses immunomagnetic beads coated with antibodies against EpCAM. Other CTC isolation modalities rely on physical characteristics such as size, density, deformability, and charge. However, because CTCs are highly heterogeneous and many CTCs exhibit a hybrid-EMP or fully mesenchymal phenotype, enrichment by a single epithelial surface marker or physical characteristic may not be sufficient to capture the full array of CTCs in the blood [7]. To overcome this limitation, techniques using multiple antibodies that mark epithelial and mesenchymal states (e.g. a combination of EpCAM, cytokeratin, and vimentin) and tumor-specific cell surface markers including HER2 and EGFR have been more effective in sampling the different populations of CTCs circulating in the blood [19]. However, these “double positive” isolation technologies

still fail to enrich for hybrid-EMP CTCs that express neither epithelial nor mesenchymal commonly examined markers [8]. Negative depletion of leukocytes with antibodies directed against white blood cells provides an efficient method to overcome the limitations posed by positive selection. These technologies rely on a permissive size-based separation to eliminate red blood cells followed by immunomagnetic depletion of white blood cells with CD45 antibodies [162–165]. They are considered negative selection because they enrich for CTCs based on known properties of the other cellular components of the blood, rather than making assumptions about CTC phenotypes that could bias the population of CTCs after isolation. The capability of each technique to isolate epithelial, mesenchymal, and hybrid-EMP CTCs is shown in Table 2.2. They each have their strengths and weaknesses, which are important to appreciate, however to date there in no universally accepted preferred method that allows comprehensive capture of all CTCs.

Table 2.2 Methods of CTC isolation and EMP recovery

Method	Name	Detection of Epithelial (E), Mesenchymal (M), or Hybrid (H) CTCs	References
Physical separation			
Size based filtration/microfluidics	Microcavity array (MCA); FAST disc; CellSieve	E = M = H	[166–168]
Density based centrifugation	Ficoll; OncoQuick	E = M = H	[169]
Size and deformability	ISET®; Celsee	M > H > E	[170, 171]
Cell surface charge	PEG	E = M = H	[172]
Density based centrifugation followed by invasion	CAM	M > E	[173]
Negative selection			
Microfluidic size based then negative selection for CD45	CTC-iChip; Cytelligen® and iFISH	E = M = H	[162, 163]
Density separation of tetrameric antibody complexes for CD45, CD66b and glycophorin	RARE	E = M = H	[164]
Density gradient separation then anti-CD45 based negative immunomagnetic enrichment	*unnamed*	E = M = H	[165]
Positive selection			
Cell surface vimentin	CSV 84-1	M	[118]
Cell surface EpCAM and FRα	*unnamed*	E = M > H	[174]
High throughput microscopy for immunofluorescence or FISH	Epic CTC Platform®; FAST	E = M > H	[175–177]
EpCAM based immunomagnetic separation	CellSearch®	E	[170]
Microfluidic size based then EpCAM based immunomagnetic separation	CTC-chip; Herringbone; eDAR; OncoBean	E	[178–181]
Sized based filtration then EpCAM, CK, vimentin, and twist RNA-ISH	CanPatrol	E = M > H	[94]
Flow Cytometry for surface epithelial markers	IE/FC	E	[182]
Filtration using selective size amplification	SSA-MOA	E	[183]
Clusters			
Size based filtration	FMSA; Cluster-Chip	M = H > E	[143, 144, 184]

2.3 Clinical Correlates and Future Study

2.3.1 EMP as a Biomarker for Progression, Aggressiveness, and Drug Selection

The functional connections between EMP and cancer progression are well-established and are supported by prognostic correlations observed in patient-derived samples. In ovarian cancer, higher EMP scores are correlated with worse prognosis, both for overall and disease-free survival [156]. In metastatic breast, pancreatic, and hepatocellular carcinomas, increases in EMP CTCs are associated with progression, poor therapeutic response, metastasis, and worse prognosis while patients responding to therapy show a decrease in EMP CTCs [7, 19, 43, 80, 93, 95, 127]. The hybrid-EMP phenotype predominates in many cancer types, including aggressive breast cancer and melanoma, and may therefore indicate a worse prognosis than tumors with a purely mesenchymal phenotype [80]. Further, cancer cells exhibiting hybrid-EMP were more plastic, and more efficient in tumor budding, invasion, stemness, CTC cluster formation, and drug resistance [34, 51]. Because CTCs are hematogenously circulating and represent many stages of metastasis, evaluation of EMP in CTCs may have clinical relevance as a biomarker [93]. However, these studies are still preliminary and the prognostic value of EMP CTCs in monitoring therapeutic resistance or progression has not been fully determined and no recommendation for clinical monitoring has been issued [127, 185].

2.3.2 Prevention or Reversal of EMP as a Therapeutic Target

In addition to serving as a biomarker, EMP may be an attractive therapeutic target to slow or halt metastasis. Current clinical trials aiming to prevent or reverse EMT are testing TGFβ inhibition (LY2157299 in glioblastoma and hepatocellular carcinoma), clusterin (a TGFβ mediator) inhibition (AB-16B5 in advanced solid tumors), platelet inhibition (aspirin in metastatic breast and colorectal cancer), AXL inhibition (TP-0903 in refractory solid tumors), and Src kinase inhibition, with mixed results [186–192]. Reversing transition to a mesenchymal state through redifferentiation could reduce invasiveness and resensitize cells to current therapies. However, there are concerns associated with therapies targeting EMP. First, MET is likely required for outgrowth at the secondary site and therefore such a treatment may actually support metastasis, possibly through reactivating dormant tumor cells [47, 83, 193–196]. Indeed, knockdown of the EMP transcription factors PRRX1 and Twist1 in breast cancer cells increased lung metastasis in mice [47]. Reciprocally, induction of Twist1 in a skin cancer model inhibited metastatic outgrowth [83]. Second, even if we could be confident that EMP inhibition would not be detrimental to the patient, the benchmarks for such a reversal are unclear. As described, EMP is not a single phenotype, but a broad array of intermediate states in different cells. It is therefore difficult to determine how far along the EMP continuum to reverse the cells, and how to achieve consistent effects in such a heterogeneous population. The best course of action will likely be different for different contexts and cancer types, further complicating the issue [7].

2.4 Controversies

Two papers published in 2015 using lineage tracing mouse models raised doubts about whether EMP is strictly necessary for metastasis in vivo (although they maintain support for a role in chemoresistance) [12, 13, 30, 197]. However, numerous papers in response to these findings have drawn on decades of research in support of a role for EMP in metastasis, pointing out that the complexity of this dynamic process – with interactions between multiple transcription factors, important intermediate and hard to detect phenotypes, and necessary plasticity between epithelial and mesenchymal states to complete the metastatic cycle – makes it very difficult to interpret

the results of a single lineage tracing model [7, 14, 15, 80, 195]. Future models of greater nuance relying on multiple EMP markers and single cell analysis will help to fully understand the role of EMP in metastasis.

2.5 Remaining Questions

Despite over 30 years of study, new and old questions remain to be addressed to clarify the role that EMP, and therefore CTCs and CTC clusters, play in metastasis. As we continue to probe further into the impact of EMP on tumorigenesis and metastasis, our increased awareness of the hybrid-EMP phenotypes exhibited by many tumor cells, but especially CTCs, will provide more insight into this process. Further studies are needed to define how many distinct subtypes there are within the continuum, how stable/plastic these subtypes are relative to each other, and whether their functional characteristics remain the same across different cancer types. This will require a collaborative decision regarding the markers of epithelial and mesenchymal phenotypes, the setting of thresholds for expression, and establishing of assays that mimic interconversion between states in patients. It will also need to be determined whether these hybrid-EMP subtypes are best modeled as a continuum or as a trans-differentiation. This will be informed by studies examining how cells transition between the subtypes, including examinations of both transcriptional and post-transcriptional regulations.

Beyond defining hybrid-EMP, it is becoming clear that hybrid-EMP in CTCs and tumor cells alike is correlated with a worse prognosis and higher metastatic potential that fully epithelial or mesenchymal cells [53, 129]. It remains to be determined whether it is the hybrid-EMP cells themselves, or just the existence of a more heterogeneous population of tumor cells, that is the cause of this observation. On the one hand, metastasis requires both mesenchymal and epithelial processes, and cells locked into a mesenchymal state may fail to initiate a tumor in the secondary site. It is possible that hybrid-EMP CTCs encompass the population of CSCs that are the crucial determinants of successful tumor reinitiation. On the other hand, different cancer types and individual cancers exhibit different levels of hybrid-EMP, and yet many cancers are metastatic. With the recent identification of CTC clusters and the appreciation of their higher metastatic potential, it is possible that heterogeneous clusters of CTCs, containing cells with epithelial, hybrid-EMP, and mesenchymal phenotypes can form and cooperatively make the metastatic journey, with the mesenchymal cells "shepherding" the epithelial cells to their destination. Our ability to address these and other mechanistic questions will be aided by technological developments. Already, CTC isolation technologies have given us an opportunity to study some of these questions in the most appropriate setting – invaded cells that are the putative precursors of metastasis. Because these rare cells must be enriched, it will be crucial to select the appropriate isolation technology so that our evaluation of the breadth of EMP phenotypes in CTCs is not biased. To confidently accomplish this, we will need to standardize epithelial, mesenchymal, and CTC markers. Upon isolation of a physiologically relevant CTC population, advances in genomics and proteomics will allow for comprehensive mapping of transcriptional, epigenetic, and post-transcriptional differences in EMP phenotypes in individual CTCs and throughout disease progression. Finally, although CTCs are the metastatically competent population, upon isolation they still provide only a snapshot in the EMP progression of that cell. As with all EMP studies, animal models and lineage tracing technologies will be crucial to visualize and ultimately understand the implications of EMP on metastasis in vivo.

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