

Epithelial–Mesenchymal Plasticity and Circulating Tumor Cells: Travel Companions to Metastases

Marie-Emilie Francart,¹ Justine Lambert,¹ Aline M. Vanwysberghe,¹ Erik W. Thompson,² Morgane Bourcy,¹ Myriam Polette,³ and Christine Gilles^{1*}

¹GIGA-Cancer, Laboratory of Tumor and Development Biology, University of Liège, Liège, Belgium

²Institute of Health and Biomedical Innovation and School of Biomedical Sciences, Queensland University of Technology, and Translational Research Institute Brisbane, and University of Melbourne Department of Surgery, St Vincent's Hospital, Melbourne, Australia

³Inserm UMR-S 903, University of Reims Champagne-Ardenne, Biopathology Laboratory, CHU of Reims, Reims, France

Epithelial–mesenchymal transitions (EMTs) associated with metastatic progression may contribute to the generation of hybrid phenotypes capable of plasticity. This cellular plasticity would provide tumor cells with an increased potential to adapt to the different microenvironments encountered during metastatic spread. Understanding how EMT may functionally equip circulating tumor cells (CTCs) with an enhanced competence to survive in the bloodstream and niche in the colonized organs has thus become a major cancer research axis. We summarize here clinical data with CTC endpoints involving EMT. We then review the work functionally linking EMT programs to CTC biology and deciphering molecular EMT-driven mechanisms supporting their metastatic competence. *Developmental Dynamics* 000:000–000, 2017. © 2017 Wiley Periodicals, Inc.

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Introduction

The hematogenous metastatic spread of epithelial tumors is a complex process involving the liberation of circulating tumor cells (CTCs) in the bloodstream, their survival in the circulation, the colonization of secondary organs as disseminated tumor cells (DTCs) and finally, after an eventual period of dormancy, growth at secondary sites and overt metastasis development.

Being easily accessible in the bloodstream, CTCs have attracted enormous attention for their potential clinical significance (Toss et al., 2014; Joosse et al., 2015; McInnes et al., 2015; Yu et al., 2015; Alix-Panabieres and Pantel, 2016; Lee et al., 2016; Masuda et al., 2016; Wang et al., 2017b). Detecting, enumerating, characterizing and understanding CTC biology may indeed help identify metastasis-predicting factors, guiding treatment decisions before the detection of overt metastases and assessing therapeutic efficacy (Krebs et al., 2014; Joosse et al., 2015; McInnes et al., 2015; Pantel and Speicher, 2015; Alix-Panabieres et al., 2017). CTCs are also considered as potential cellular therapeutic targets (Li and King, 2012; Li et al., 2015a, 2016).

The comprehension that CTCs constitute a genetically and phenotypically very heterogeneous population has further stimulated studies aiming to characterize the metastatic founders within the CTC population. It has thus become a major cancer research axis to phenotype CTCs, to identify premetastatic subsets and to

unravel the molecular mechanisms enabling some of them to accomplish the early steps of the metastatic colonization (i.e., survival in the bloodstream and early seeding in colonized organs) (Nadal et al., 2013; Krebs et al., 2014; Pantel and Speicher, 2015).

We have reviewed here literature data on how epithelial–mesenchymal transitions (EMTs) may impact CTC biology by generating hybrid phenotypes with mesenchymal attributes (mesenchymally-shifted cells) that may favor their liberation and survival in the bloodstream, and their metastatic seeding.

Epithelial–Mesenchymal Plasticity/Epithelial–Mesenchymal Transition

Epithelial–mesenchymal plasticity (EMP) (Thompson et al., 2005; Thompson and Haviv, 2011; Savagner, 2015; Ye and Weinberg, 2015; Chaffer et al., 2016; Nieto et al., 2016) is today considered a central actor of the metastatic cascade, providing tumor cells with the ability to adapt to different microenvironments met during their translocation to colonized organs (i.e., adjacent stroma, blood, newly colonized organs). Timely and spatially regulated dynamic interconversions between epithelial states and states that are more mesenchymal indeed occur throughout the metastatic cascade, enabling tumor cells to survive/develop in successively encountered microenvironment. Schematically (Figs. 1 and 2), the classical view of EMP implication to the metastatic cascade

*Correspondence to: Christine Gilles, Laboratory of Tumor and Development Biology (LBTD), GIGA-Cancer, Pathology Tower, B23, CHU Sart-Tilman, University of Liège, Liège 4000, Belgium.
E-mail: cgilles@ulg.ac.be

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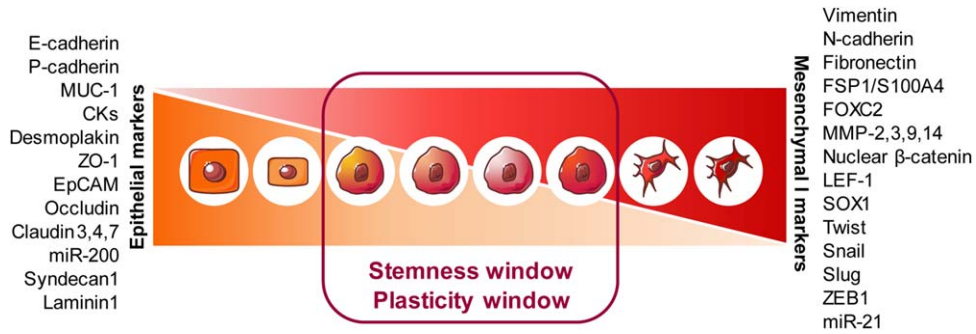


Fig. 1. Hybrid phenotypes along the epithelial (E) -to-mesenchymal (M) spectrum.

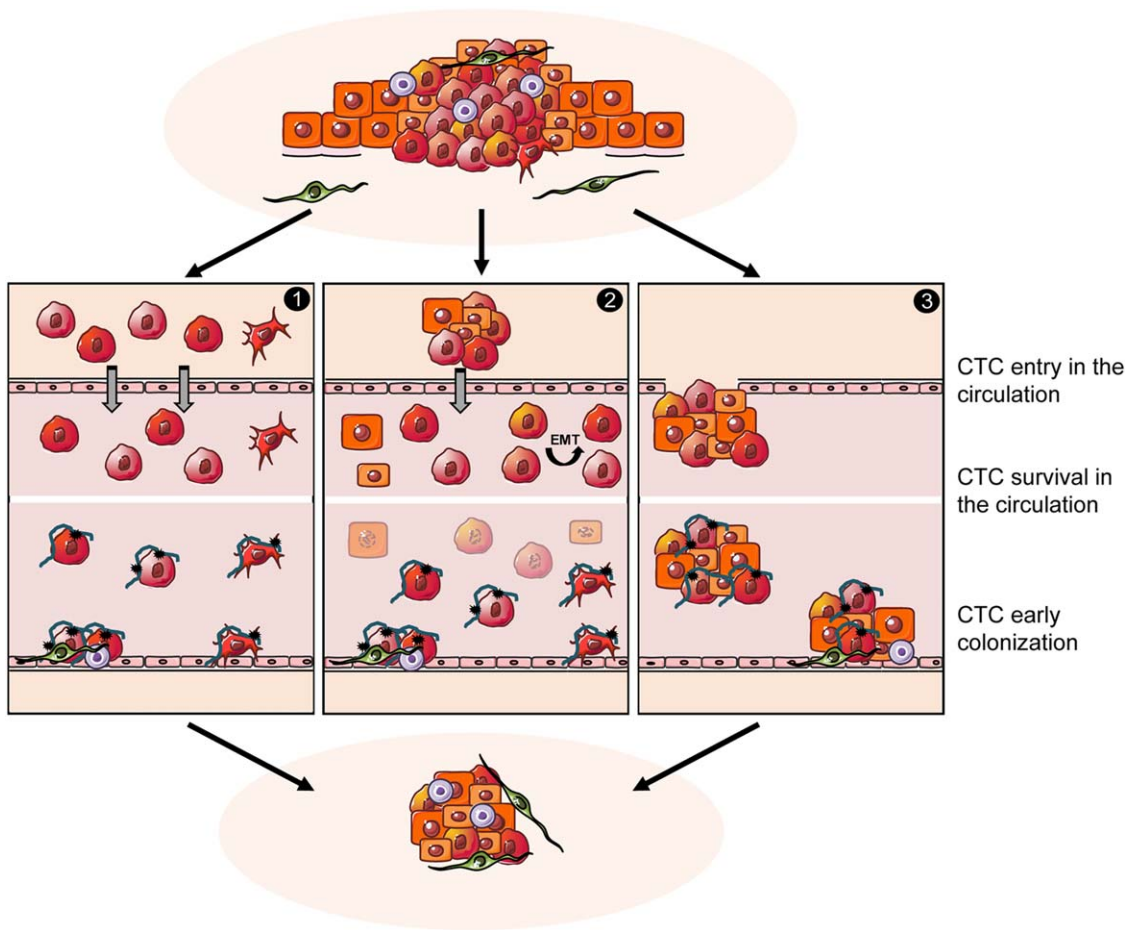


Fig. 2. Schematic nonexclusive hypotheses of epithelial–mesenchymal transition’s (EMT) contribution to the biology of CTC during the metastatic spread. ❶ EMT occurs in the primary tumors providing tumor cells with enhanced invasive properties facilitating active intravasation and the liberation of mesenchymally-shifted circulating tumor cell (CTC) phenotypes. Displaying enhanced EMT-driven survival properties (i.e., activation of survival pathways, activation of local coagulation, microtentacle formation, or evasion from immune checkpoints), those mesenchymally-shifted CTCs are able to accomplish the early colonization phases. Among the EMT-derived CTCs that have niched in colonized organs, those belonging to the plasticity window will then be able to revert to a more epithelial state thought to be involved in the metastatic outgrowth. ❷ EMT occurs in the primary tumors, EMT-derived cells intravasate, bringing with them more epithelial intermediates through a cooperative process (which may eventually undergo a EMT within the blood stream, for instance by means of transforming growth factor-beta liberated from platelets). The more epithelial CTCs expressing low survival properties are submitted to shear stress, anoikis, or cytotoxic immune attack and are eliminated. As in scheme 1, mesenchymally-shifted CTCs survive and succeed in the early colonization phases. ❸ Clusters of CTC gain the circulation through corrupted blood vessels, minimizing the effect of anoikis, thus promoting the survival of more epithelial phenotypes in the bloodstream (the presence of mesenchymally-shifted cells in these clusters may further support the survival of CTCs within the clusters). Clusters of CTC may form niches, allowing those with metastatic competence to develop overt metastases. Platelets = , Fibrin = , Recruited stromal cells = .

involves sequential EMTs followed by mesenchymal–epithelial transitions (METs) at different body locations (Tsai and Yang, 2013; Jolly et al., 2015a; Liu et al., 2015; Beerling et al., 2016; Celia-Terrassa and Kang, 2016; Chaffer et al., 2016; Diepenbruck and Christofori, 2016; Kolbl et al., 2016).

Thus, a switch toward a more mesenchymal state (EMT) is considered to contribute to the first phases of the metastatic translocation, i.e., tumor invasion, intravasation, liberation of CTCs and survival in the bloodstream, and metastatic niche formation. Conversely, a reversed MET process has been associated with an enhanced ability to proliferate and develop overt metastases in secondary organs, thus contributing to the deadly late stages of metastatic development (Gunasinghe et al., 2012). Unlike developmental EMT, tumor-associated EMT has rarely been reported to involve a complete lineage switching, but rather the generation of intermediate states (hybrid phenotypes) that distribute along the epithelium (E) to mesenchymal (M) continuum (as schematically represented in Fig. 1). Phenotypic plasticity is believed to be restricted to certain hybrid phenotypes also endowed with stem cell characteristics [overlapping to some extent to the so-called cancer stem cell (CSC) population] (Jolly et al., 2015a).

Importantly, EMT programs have been shown to directly induce stem cell properties in epithelial tumor cells (Mani et al., 2008; Morel et al., 2008; Bhat-Nakshatri et al., 2010; Ansieau, 2013; Kotiyal and Bhattacharya, 2014; Mallini et al., 2014; Schmidt et al., 2015; Mladinich et al., 2016). Thus, EMT and CSC characteristics have been shown to overlap to some extent both in *in vitro* cell systems but also in tumors and CTCs from cancer patients (Aktas et al., 2009; Giordano et al., 2012; Kasimir-Bauer et al., 2012; Ksiazkiewicz et al., 2012; Barriere et al., 2014; Bock et al., 2014; Krawczyk et al., 2014; Tinhofer et al., 2014). Sometimes referred to as “metastable” phenotypes (Klymkowsky and Savagner, 2009; Savagner, 2015), these hybrid phenotypes with mesenchymal attributes and CSC markers would be more efficient for metastasis. Levine and co-workers have recently shown with elaborate modeling that the hybrid state may be quite stable (Jolly et al., 2015a,b, 2016).

Although the successive EMT/MET scheme of the metastatic spread presented above is generally accepted, it is still a subject of debate and discussions (Tsuiji et al., 2009; Brabletz, 2012; Fischer et al., 2015; Zheng et al., 2015; Diepenbruck and Christofori, 2016). It indeed remains unclear if the same plastic tumor cell is able to overcome all obstacles of the metastatic cascade through phenotypic adaptation or whether further genetic alterations occur during the metastatic cascade that empower some tumor cells to form metastases. Whether different phenotypes along the E to M continuum cooperate throughout the metastatic process to protect cells with the highest ability to proliferate at secondary site is also a possibility. Two recent studies using transgenic models suggested that EMT is dispensable for metastasis but required for chemoresistance and further revived the debate (Fischer et al., 2015; Zheng et al., 2015). It is also very likely that all these mechanisms may coexist to different extents, depending on the model used or the cancer type analyzed, making our understanding of their roles in the metastatic process and the establishment of effective therapies more difficult.

EMP associated with metastatic progression is recognized to be commonly regulated by molecular actors of core EMT programs, although a full mesenchymal conversion is considered uncommon. These EMT core molecular actors have been reviewed elsewhere (Kalluri and Weinberg, 2009; De Craene and Berx, 2013;

Tsai and Yang, 2013; Lamouille et al., 2014; Jolly et al., 2015b; Nieto et al., 2016) and will, therefore, not be detailed here. EMT associated with metastasis is nevertheless clearly not a well-defined unique molecular program driving a binary transformation from a fully epithelial cell to a mesenchymal cell, nor does it involve the generation of well-characterized sequential hybrid phenotypes. There is indeed a wide repertoire of epithelial plasticity and a multiplicity of potential hybrid phenotypes. There is thus a clear need to identify how specific molecular actors of EMT programs may functionally impact on specific stages of the metastatic cascade to further refine therapeutic strategies.

Examining the relationship between EMT and CTCs has gained a fast-growing interest in the past decade. EMT has indeed rapidly emerged as a process endowing tumor cells with properties that may functionally impact CTC life cycle (Bonnomet et al., 2010; Barriere et al., 2014; Krawczyk et al., 2014; Aceto et al., 2015; Jolly et al., 2015a; Liu et al., 2015; McInnes et al., 2015; Pantel and Speicher, 2015; Kolbl et al., 2016; Alix-Panabieres et al., 2017), including invasive/motile properties (Nieto et al., 2016), resistance to apoptosis/anoikis (Tiwari et al., 2012; Frisch et al., 2013; Cao et al., 2016), and stemness properties (Ombrato and Malanchi, 2014; Ye and Weinberg, 2015; Fabregat et al., 2016).

EMT Facilitates the Entry of CTCs into the Circulation

Because EMT has been extensively shown to confer migratory and invasive properties to epithelial tumor cells, it is generally considered that mesenchymally-shifted tumor cells display enhanced ability to actively enter the circulation and thus become CTCs (Bonnomet et al., 2010; Tsai and Yang, 2013; Chiang et al., 2016). Accordingly several experimental settings using transendothelial migration assays, chick chorioallantoic membrane (CAM) assays, and intravital imaging emphasized a contribution of EMT (mainly using cells with forced expression of EMT transcription factors such as Snail or ZEB1) in intravasation (Drake et al., 2009; Ota et al., 2009; Bonnomet et al., 2010). A crucial role of EMT-induced proteases has also been identified. Using intravital imaging on tumor cells xenografts, it has for instance been shown that the activation of the transforming growth factor- β (TGF- β) pathway associates with a certain degree of trans-differentiation, promotes single cell motility, and enables invasion into blood vessels. The activation of the TGF- β pathway was shown to be a transient event and was not maintained at distant sites (Giampieri et al., 2009). This is also in agreement with several earlier studies associating EMT with a single-cell mode of migration (Friedl and Gilmour, 2009).

Other mechanisms of entry of tumor cells in the circulation have also been proposed. Thus, cooperative processes by which EMT shifted cells would “help” more epithelial tumor cell phenotypes (suggested to be more competent for metastasis) to gain the circulation may also occur and have also been reported in a syngenic tumor model (Tsuiji et al., 2008, 2009). Indeed, Tsuiji et al. demonstrated that EMT negative hamster keratinocytes (HPCP-1) were unable to metastasize after subcutaneous injection unless co-injected with EMT-transformed counterparts (overexpressing the downstream effector of the TGF- β pathway p12^{CKD2-AP1}) that enabled intravasation of the EMT-negative cells. Another study similarly reported a cooperation between EMT- and EMT+ cells for metastasis in xenograft models of human prostate and

bladder cancer cell lines in which the expression of Snail was modulated (Celia-Terrassa et al., 2012).

In line with the observation of corrupted blood vessels in tumors, a passive mode of entry of tumor cells in the circulation, that could thus be EMT-dependent or EMT-independent, has also been suggested (Alpaugh et al., 2002; Bockhorn et al., 2007; Bednarz-Knoll et al., 2012). Such a passive mode of entry has been more particularly advocated to explain the detection of clusters of CTCs in the blood of cancer patients, although a collective migration process during intravasation cannot be excluded (Giampieri et al., 2010; Aceto et al., 2015). Clusters of CTCs had actually been observed as early as in the 1970s (Fidler, 1973; Liotta et al., 1976). The possibility that these clusters might actually form during the processing of the blood samples during the CTC assay has been debated (Bednarz-Knoll et al., 2012), although they are much less prevalent than isolated CTCs. These clusters are today recognized as functional entities and have been detected in various types of cancers including breast, lung, pancreas, prostate, or kidney cancers (Molnar et al., 2001; Stott et al., 2010; Cho et al., 2012; Hou et al., 2012; Krebs et al., 2012; Yu et al., 2013; Aceto et al., 2014; Mu et al., 2015; Paoletti et al., 2015; Wang et al., 2017a).

Adding to this, a shift toward a more mesenchymal phenotype may also be gained within the circulation. For instance, Labelle et al. suggested that TGF- β liberated from activated platelets may enhance EMT in TGF- β -responsive tumor cells and promote metastasis formation (Labelle et al., 2011; Pantel and Speicher, 2015).

CTCs may thus enter the circulation either as mesenchymally-shifted cells or not but may also acquire mesenchymal attributes within the circulation (Fig. 2). Reflecting the likelihood of coexisting mechanisms used by tumor cells to enter the bloodstream, and adding genomic heterogeneity, CTCs are indeed a phenotypically heterogeneous population. Although the active contribution of EMT implication to the different processes of entry of CTCs in the circulation may vary, the repetitive observation of CTCs expressing mesenchymal attributes both in the blood of tumor animal models or in the blood of cancer patients (Table 1) clearly support a contribution of EMT to CTC phenotypical heterogeneity (Bednarz-Knoll et al., 2012; Krawczyk et al., 2014; Liu et al., 2015; Pantel and Speicher, 2015; Kolbl et al., 2016; Alix-Panabieres et al., 2017).

Regarding animal models, Rhim and coworkers detected CTCs expressing the EMT transcription factor ZEB2 at the premalignant stage of tumor progression in a K-Ras-driven mouse pancreatic tumor model (Rhim et al., 2012). Using a transgenic mouse model expressing Twist under an inducible keratin 5 promoter, Tsai and colleagues showed that Twist induction increased the number of CTCs and that these CTCs presented an EMT phenotype with a loss of E-cadherin and a gain of vimentin expression (Tsai et al., 2012). Using a xenograft system of human breast tumor MDA-MB-468 cells, we also reported dynamic EMT changes in the primary tumors and the liberation of CTCs expressing EMT markers, including Snail, Slug, and vimentin (Bonnomet et al., 2012). In these different studies, the liberation of EMT-shifted CTCs correlated with the appearance of metastatic lesions, supporting the idea that these CTCs expressing mesenchymal traits could be metastatic founders.

Similarly, CTCs expressing common EMT actors such as EMT transcription factors (ZEB1, Twist, Snail, or Slug), vimentin, or N-cadherin have been observed in different types of cancers and particularly in breast cancer patients but EMT-shifted CTCs were

also identified in patients with lung, colorectal, prostate, bladder, or endometrial cancers (Table 1).

In light of these observations, it has been proposed that examining mesenchymal markers should be more systematically included in the detection of CTCs (Bednarz-Knoll et al., 2012; Barriere et al., 2014; Bulfoni et al., 2016b). Although CTC isolation techniques have been extensively reviewed elsewhere and will not be detailed here, it is important to perceive that the potential contribution of EMT to CTC biology largely complicates their purification and characterization (Bednarz-Knoll et al., 2012; Alix-Panabieres and Pantel, 2014; Joosse et al., 2015; Pantel and Speicher, 2015; Hyun et al., 2016; Alix-Panabieres et al., 2017). Indeed, CTC isolation and identification techniques are most commonly based on the detection of epithelial markers (i.e., EpCAM is frequently used in antibody-based purification procedure and cytokeratins are often examined to discriminate epithelial CTCs from blood cells). It has thus been recognized that certain mesenchymally-shifted CTCs could be excluded from different CTC assays. This has stimulated the development of technologies to include the detection of EMT-shifted CTCs and their more systematic examination (Bednarz-Knoll et al., 2012; Barriere et al., 2014; Bulfoni et al., 2016b). Strategies, essentially based on physical properties of CTCs (mostly size-based technologies) and using filtration or microfluidic device, and/or negative selection of blood cells, are thus being developed that do not rely on epithelial marker for CTC isolation, thus enriching untagged CTCs (Aceto et al., 2015; Alix-Panabieres et al., 2017).

EMT Enhances CTC Metastatic Competence

Considering that CTCs expressing mesenchymal attributes are commonly found in cancer patients in the light of the prometastatic properties provided by EMT programs, it has been suggested that these cells exhibiting hybrid phenotypes within the CTC population might be metastatic founders, so called MICs (metastasis initiating cells).

In accordance with this hypothesis, the presence of mesenchymally-shifted CTCs has been associated with poor clinical parameters in several studies as detailed in Table 1. Among the most frequently examined EMT markers in CTCs are EMT transcription factors (Twist, ZEB1, Snail, or Slug), vimentin, fibronectin, N-cadherin, PAI-1 (plasminogen activator inhibitor-1), c-MET (HGF receptor), or molecular actors of survival pathways (epidermal growth factor receptor [EGFR], Akt, PI3K). Regarding epithelial markers, E-cadherin and cytokeratins are commonly investigated. In the light of the finding that EMT features often correlate or even induce the expression of stem cell markers, CD44, ALDH1 or CD133 (promin 1) are also frequently examined. Thus, it was for instance shown that CTCs expressing the functional stem cell marker ALDH1A1 together with the EMT transcription factor Twist were more frequently detected in patients with metastatic breast cancer (Papadaki et al., 2014). Another study identified Plastin 3 as a good marker of EMT-shifted CTCs, which were shown to harbor a prognostic relevance (Yokobori et al., 2013). Bulfoni and coworkers also associated EMT traits in CTCs with poor prognosis in metastatic breast cancer patients (Bulfoni et al., 2016a).

The prevalence of mesenchymally-shifted CTCs is, however, hard to establish, varying with the tumor type, the stage of the disease and the markers analyzed. In an elegant study, Yu et al. (2013), examined several epithelial (Keratins 5, 7, 8, 18, 19,

TABLE 1. Detection of EMT and Stem Cell Markers in CTCs from Cancer Patients

EMT and stemness markers (+ others associated)	Type of tumor	Method of separation/characterization	Method of detection	Nr of patients	Correlation between EMT markers and clinical parameters	Ref
EGFR, pEGFR, HER2, pAkt, pPI3K	Breast cancer	Ficoll density gradient/EpCAM positive CELLlection beads/IF CK	IF	38	Metastases [EGFR and pPI3K]	(Kallergi et al., 2008)
Twist, PI3K α , Akt2, ALDH1	Beast cancer	AdnaTest BreastCancer	RT PCR	39	Therapy resist [CTC ^{EMT+/ALDH1}]	(Aktas et al., 2009)
ALDH1, CD44	Breast cancer (M ₁)	Ficoll density gradient/IF CK-CD24	IF	30	ND	(Theodoropoulos et al., 2010)
Vim, FN	Breast cancer (M ₁)	EpCAM positive CELLlection beads/RT PCR CD45-CK	RT PCR	55	↓ FPS	(Gradilone et al., 2011)
Vim, E-cad, N-cad, CKs	Lung cancer (M ₀ and M ₁)	ISET (filter-based size exclusion approach)/IHC CD45	IHC	7	ND	(Hou et al., 2011)
Vim, Twist	Breast cancer (M ₀ and M ₁)	Ficoll density gradient/CD45 negative Dynal CELLlection beads/IF CK	IF	50	Metastases	(Kallergi et al., 2011)
Vim, CKs	NSCLC (M ₁)	ISET (filter-based size exclusion approach)/Identification by a cytopathologist	IF	6	ND	(Lecharpentier et al., 2011)
Vim, FN, ALDH1	Breast cancer (multi stages)	EpCAM positive CELLlection Dynabeads/RT PCR CD45-CK	RT PCR	92	Stage disease	(Raimondi et al., 2011)
Twist, Snail, Slug, ZEB1, FoxC2	Breast cancer (M ₀)	CellSearch, AdnaTest Breast Cancer, Ficoll density gradient + CD45 magnetic beads depletion	RT PCR	52	Neoadjuvant therapies resist [CTC ^{EMT+}]	(Mego et al., 2011)
Vim, E-cad, N-cad, O-cad, CD133	CRPC (M ₁) Breast cancer (M ₁)	CellSearch	IF	57	ND	(Armstrong et al., 2011)
Twist, PI3K α , Akt2, ALDH1	Breast cancer (M ₀)	AdnaTest Breast Cancer	RT PCR	130	No correlation found	(Barriere et al., 2012b)
Twist, PI3K α , Akt2, ALDH1, Bmi1, CD44	Breast cancer	AdnaTest EMT-1/Stem Cell	RT PCR	61	Negative metastases lymph node status [ddCTC]	(Barriere et al., 2012a)
Twist, Snail, ZEB1, TG2	Breast cancer (M ₁ HER2+)	CellSearch	RT PCR	28	ND	(Giordano et al., 2012)

TABLE 1. Continued

EMT and stemness markers (+ others associated)	Type of tumor	Method of separation/characterization	Method of detection	Nr of patients	Correlation between EMT markers and clinical parameters	Ref
Twist, PI3K- α , Akt2, ALDH1	Breast cancer	AdnaTest Breast Cancer	RT PCR	502	No correlation found	(Kasimir-Bauer et al., 2012)
CD44, CD47, c-MET	Breast cancer (M ₀ luminal)	CellSearch RosetteSep selection kit hematopoietic cells depletion/ FACS EpCAM -CD45-PI	FACS	350	↓ OS [CTC _S ^{CD44+MET+CD47+}] Metastatic [CTC _S ^{CD44+MET+CD47+}] Disease progression	(Baccelli et al., 2013)
84 EMT-related genes tested	Prostate cancer	ScreenCell Selection (filter-based size exclusion approach)/IF CD45 + micromanipulator	qRT PCR chip assay	8	Castration treatment resist [markers CTC ^{EMT+}]	(Chen et al., 2013)
Vim, Twist	Hepatocellular carcinoma	Ficoll density gradient/Asialofetuin biotinylation magnetic beads positive selection/IF CD45-Hepatocyte-Specific Antigen	IF	60	Tumor size, TNM, Milan criteria [vim] Portal vein tumor thrombosis [vim, Twist]	(Li et al., 2013)
Plastin3	Colorectal cancer	Ficoll density gradient/CD45 magnetic beads depletion/EpCAM magnetic beads positive selection/IF CK-vim	IF	771	↓ OS	(Yokobori et al., 2013)
CKs 8,19, TFF1/ TFF3, PAI-1, FN1, ECM, WNT, integrin, interleukin receptors, TGF- β , interleukin-6, mammaglobins	Breast cancer	Herringbone-chip EpCAM, HER2, EGFR positive selection	FISH	41	Disease progression [CTC ^{M+}] Therapy response [reversible shifts between CTC ^{E+} /CTC ^{M+}]	(Yu et al., 2013)
Snail, ZEB1, ZEB2, ETV5, Notch1, TGF β 1, ALDH1, CD44	Endometrial cancer	CELLlection epithelial enrich/qRT PCR CD45	qRT PCR	34	High risk endometrial cancer [CTC ^{high plasticity}]	(Alonso-Alconada et al., 2014)
ZEB2, LOXL3, VIL1 TIMP1, CLU	Colorectal cancer	CELLlection epithelial enrich/qRT PCR CD45	qRT PCR	50	↓ PFS/OS	(Barbazan et al., 2014)

TABLE 1. Continued

EMT and stemness markers (+ others associated)	Type of tumor	Method of separation/characterization	Method of detection	Nr of patients	Correlation between EMT markers and clinical parameters	Ref
Twist, Snail, Slug, ZEB1, CK19	Breast cancer (M0)	RosetteSep selection kit CD45 negative selection	qRT PCR	149	High grade tumor ↑ KI67	(Cierna et al., 2014)
Vim, CD44, seprase, methyl group to CpG sites	CRPC (M1)	Vitax's rare cell enrichment technology: hematologic lineage markers depletion, EpCAM, PSMA, tumor/stem cell markers positive selection or CellSearch	IHC, FACS, DNA methylation array	23	ND	(Friedlander et al., 2014)
Vim, Snail, uPAR	Breast cancer (M0)	Density gradient centrifugation/CD45 magnetic particles depletion	qRT PCR IF	117	Lymph nodes metastases	(Markiewicz et al., 2014)
Twist, ALDH1	Breast cancer (M0 and M1)	Ficoll density gradient/IF CD45, CKs	IF	130	Metastases [ALDH1 ^{high} /Twist ^{high} nuclear]	(Papadaki et al., 2014)
Vim, Slug, EGFR	Breast cancer (M0)	Carcinoma Cell Enrichment and Detection kit, MACS technology CKs magnetic beads positive selection/IF CD45	IF	78	↓ PFS after receiving systemic treatment [CTCs ^{CK-EGFR+}] Tumor size [vim, Slug]	(Serrano et al., 2014)
Ratio Vim/CKs	Breast cancer (M0 and M1)	CellSearch	IF	61	↓ OS [CTCs ^{Epcam+ CK low}]	(Polioudaki et al., 2015)
EpCAM, CKs 8, 18, 19, Vim, Twist	Gastric cancer	CanPatrol system (filtration system)/FISH CD45	FISH	44	Therapy response [CTC ^{M+}]	(Li et al., 2015b)
Cell surface vimentin (84–1 vimentin Ab)	Colorectal cancer (M1)	CD45 depletion/84–1 positive cell selection	FISH	101	Therapy response	(Satelli et al., 2014)
EpCAM, CKs 8, 18, 19, Vim, Twist	NSCLC, liver, breast, gastric and nasopharyngeal cancer	CD45 depletion/CanPatrol (filtration system)/IF-FISH CD45	FISH IF	164	Metastases [CTC ^{M+}]	(Wu et al., 2015)
EpCAM, E-Cad, N-Cad, CD44, CD146	Breast cancer (M1)	CD45 MicroBeads depletion/DEPArray sorting CD45 negative cells	RT PCR	56	↓ PFS/OS [CTC ^{EM+}] Tumor type, disease progression, proliferation, metastases site [CTC ^{ab}]	(Bulfoni et al., 2016a)

TABLE 1. Continued

EMT and stemness markers (+ others associated)	Type of tumor	Method of separation/characterization	Method of detection	Nr of patients	Correlation between EMT markers and clinical parameters	Ref
Vim, c-MET, HER3, ERCC1, JAG1	NSCLC	CellSearch system, Adna-EMT-2 test + multiplex RT-PCR vs qRT PCR, Ficoll density gradient + IF CK CD45 ALDH1	qRT PCR	62	ND	(Hanssen et al., 2016)
55 breast cancer associated genes	Breast cancer (M1)	AdnaTest BreastCancer	qRT PCR	147	*↓ RFS [RAD51] *↓ OS [CD24, HDAC2, KRAS, PIK3CA, RAD51]	(Hensler et al., 2016)
EpCAM, CKs7, 8	Breast cancer (M1)	Parallel multi-orifice flow fractionation chip (p-MOFF system)/IF CD45-CK	IF	32	ND	(Hyun et al., 2016)
Ki67, Vim	CRPC (M1)	CellSearch	IF	93	↓ OS	(Lindsay et al., 2016)
EpCAM, CKs 8, 18, 19, Vim, Twist	Hepato cellular carcinoma	CD45 depletion/CanPatrol CTC enrichment technique (filtration system)/FISCH CD45	FISH	33	Extra hepatic metastases [CTC ^{M+}] Intra hepatic metastases [CTC ^{EMT+}] Tumor size [CTC ^{E+}]	(Liu et al., 2016)
Twist, PI3K α , Akt2, ALDH1	Colorectal cancer (M1)	CD45 Dynabeads M-450 depletion/Dynabeads Epithelial Enrich/qRT PCR survivin-CK	qRT PCR	78	↓ PFS/OS [Akt2]	(Ning et al., 2016)
Twist, PI3K α , Akt2, ALDH1	Bladder Cancer	AdnaTest EMT2/SC system	PCR	83	Clinical stage	(Todenhofer et al., 2016)
EpCAM, CK8,18,19, Vim, Twist, Akt2 and Snail	Colorectal cancer	CD45 depletion/CanPatrol CTC enrichment technique (filtration system)/FISCH CD45	FISH	1203	Clinical stage, lymph node and distant metastases [CTC ^{M+} and E ^{+/M+}]	(Zhao et al., 2016a)

Vim, vimentin; FN, fibronectin; CKs, cytokeratins; Cad, cadherin; Ab, antibody; M₀, non metastatic; NSCLC, non small cell lung cancer; CRPC, castror resistant prostate cancer; PSMA, prostate specific membrane antigen; FACS, fluorescence activating cells sorting; IF, immunofluorescence; HIS, immunohistochemistry; FISH, fluorescence in situ hybridization; PI, propidium iodide; (q)RT PCR, (quantitative) reverse transcription polymerase chain polymerization; OS, overall survival; PFS, progression free survival; RFS, relapse free survival; TNM, tumor nodes metastases; ddCTC, dedifferentiated circulating tumor cells; CTC, circulating tumor stem cells; ND, not determined; *The Cancer Genome Atlas data set.

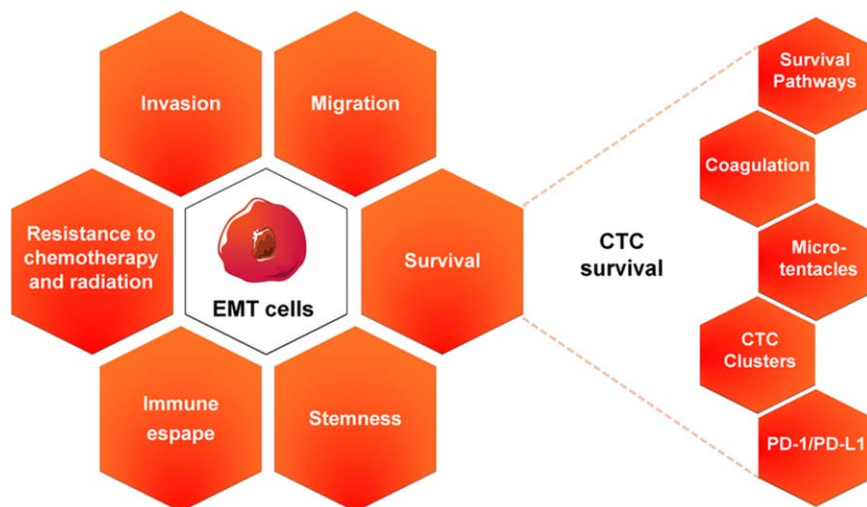


Fig. 3. Main epithelial-mesenchymal transitions (EMT)-driven properties that likely contribute to circulating tumor cells (CTC) biology. Those properties providing CTC with enhanced survival potential are more particularly discussed in the text.

EpCAM, E-cadherin) and mesenchymal (N-cadherin, fibronectin, PAI-1) markers as probe pools in CTCs from breast cancer patients. They provided evidence of EMT both in rare cells within primary tumors and more abundantly in CTCs and further arranged CTC phenotypes along the E to M spectrum into five categories. In 17 breast cancer patients analyzed, 12 displayed more than 50% of CTCs with mesenchymal attributes. Only 4 patients had a higher percentage of epithelial CTCs than mesenchymal CTCs. The percentage of mesenchymal CTCs was the highest (between 80 and 100%) in the aggressive triple negative subtype of breast cancers. Serial monitoring of 11 patients also revealed an increase in the percentage of mesenchymal CTCs, leading the authors to suggest an association between these CTCs and disease progression. Comparing blood samples performed in cancer patients before and after therapy, the authors further revealed that patients who had progressive disease while on therapy displayed an increased number of mesenchymal CTCs in the post-treatment sample (Yu et al., 2013).

Although it is feasible that the hybrid state has become stably encoded through genetic/epigenetic selection, it seems likely that the frequent detection of hybrid CTC phenotypes associated with poor clinical parameters also reflects dynamic plasticity and higher ability of hybrid E/M phenotypes to better adapt to a selective environmental context. In support of this, although the authors did not specifically examine EMT markers, phenotypical interconversion between HER2+ and HER2- CTC subpopulations isolated from breast cancer patients, demonstrate the existence of a dynamic plasticity within the CTC population (Jordan et al., 2016).

In support of these data, functional observations further suggest that specific mesenchymal attributes may support CTC metastatic competence. Thus, adding to EMT-driven invasive properties that may contribute to CTC liberation from tumor masses, EMT is also likely to enhance CTC survival in the bloodstream and in colonized organs, and also to favor early metastatic niche formation.

EMT Increases CTC Survival and Early Colonization

During translocation in the bloodstream and the initial hours of metastatic colonization, CTCs are confronted to harsh selective

pressure: they have to resist shear stress, modifications of cell-cell and cell-matrix contacts (inducing the specialized cell death program anoikis), and cytotoxic immune attack (particularly implicating natural killers, NK cells). Accordingly, several works identified a large number of apoptotic CTCs in cancer patients (Larson et al., 2004; Rossi et al., 2010) and, in some studies, a low percentage of apoptotic CTCs has been associated with poorer clinical parameters (Kallergi et al., 2013; Spiliotaki et al., 2014). For instance, Kallergi et al. detected apoptotic CTCs in patients with breast cancer irrespective of their clinical status, although the incidence of detection was higher in early compared with metastatic patients (Kallergi et al., 2013).

The mechanisms used by certain CTCs to survive in the bloodstream and during initial metastatic colonization are being better understood, and a role of EMT in conveying these specific properties is being recognized. EMT-associated properties endowing CTCs with enhanced survival/early colonizing potential may include the activation of survival pathways, the activation of the coagulation cascade, the formation of particular cytoskeletal structures (microtentacles), the evasion of particular immune checkpoints and cluster formation (Fig. 3). The contribution of EMT in supporting these mechanisms is discussed below.

Induction of Survival Pathways

An EMT-driven gain of resistance to apoptosis/anoikis has been widely reported in many cellular tumor cell systems (Bonnomet et al., 2010; Tiwari et al., 2012; Bonavida et al., 2013; Cao et al., 2016). For instance, silencing or enhancing the core transcription factors of EMT Snail, Slug, or Twist, have been shown to enhance or reduce resistance to apoptosis induced by various means (e.g. irradiation, cytotoxic drugs, serum deprivation, . . .) and in many tumor cell types (Kajita et al., 2004; Zhang et al., 2007; Escriva et al., 2008; Haddad et al., 2009; Kurrey et al., 2009; Vichalkovski et al., 2010; Zhang et al., 2010; Lim et al., 2013; Mariano et al., 2015; Roberts et al., 2016). In several studies, the positive impact of EMT on resistance to anoikis was more particularly examined (Onder et al., 2008; Smit et al., 2009; Howe et al., 2011; Kumar et al., 2011; Bao et al., 2013; Huang et al., 2013). Among others, the observed effects of EMT-induced resistance to apoptosis/

anoikis include the activation of the Akt survival pathway, a diminution of caspase activity, an induction of Bcl2 and antagonizing of p53 activity. Along these lines, molecular actors of survival pathways (e.g., EGFR, Akt, PI3K, . . .) have been detected in CTCs, sometimes in association with more classical EMT markers such as EMT transcription factors or stem cell markers (Aktas et al., 2009; Kallergi et al., 2011; Barriere et al., 2012a,b; Kasimir-Bauer et al., 2012; Hanssen et al., 2016; Todenhofer et al., 2016) (Table 1).

Expectedly and interestingly, the ability of EMT to induce tumor resistance to chemo or targeted therapies (resistance to EGFR inhibitors has been most often examined) has become a major research axis and this mechanism has been demonstrated in a variety of *in vitro* (Zhang et al., 2007, 2010; Haddad et al., 2009; Lim et al., 2013; Mariano et al., 2015; Roberts et al., 2016; Zhao et al., 2016b) and *in vivo* models (Fischer et al., 2015; Zheng et al., 2015), and also clinically (Thomson et al., 2005; Yauch et al., 2005; Du and Shim, 2016). More particularly, CTCs expressing mesenchymal markers have been associated with a resistance to chemo or targeted therapies (Mitra et al., 2015). This has been illustrated in different animal models (Fischer et al., 2015; Zheng et al., 2015) but also in cancer patients. For instance, Mego et al. reported that CTCs expressing EMT transcription factors (Twist1, Snail, Slug, ZEB1, and FOXC2) were examined in their study) in breast cancers are more resistant to neoadjuvant therapy (Mego et al., 2011). EMT and the stem cell marker ALDH1 on CTCs might also serve as an indication for therapy resistant tumor cell population (Aktas et al., 2009). An incremental expression of EMT-related genes in CTCs has also been associated with metastatic castration-resistant prostate cancers (Chen et al., 2013). It has thus been proposed that CTCs with EMT attributes could better resist chemo- or targeted therapy and would thus be related to relapse occurring after a period of remission.

Activation of Coagulation

Interestingly, increasing data also suggest an implication of coagulation actors in enhancing the ability of CTCs to survive and accomplish early metastatic colonization. The activation of coagulation has indeed long been correlated with malignancy and the beneficial impact of anticoagulants on cancer progression has been demonstrated in animal models and evaluated in clinical studies (Lee, 2010; Degen and Palumbo, 2012; Gil-Bernabe et al., 2013). The presence of CTCs has also been associated with increased risk of thromboembolism in metastatic breast cancer (Mego et al., 2009). More specifically, tissue factor (TF), a cell-associated activator of the coagulation cascade expressed by a variety of tumor cells, has emerged as a central player linking coagulation and cancer (Palumbo, 2008; Garnier et al., 2010; Ruf, 2012; Williams and Mackman, 2012; Cole and Bromberg, 2013; Gil-Bernabe et al., 2013; Ünlü and Versteeg, 2014).

Coagulation-dependent effects of TF on early survival and early metastasis have been demonstrated by the laboratory of Pr. Degen, who injected intravenously tumor cells modified for TF expression in mice with genetic defects in distal hemostatic factors (prothrombin and fibrinogen). They thus revealed that TF supports early metastasis through mechanisms dependent on these distal hemostatic factors (Palumbo and Degen, 2007; Palumbo et al., 2007; Palumbo, 2008; Degen and Palumbo, 2012). Recently, we linked TF expression and EMT and emphasized the importance of this regulatory axis on CTC survival (Bourcy et al.,

2016). We indeed observed that growth factor induced-EMT triggers TF expression in several cell systems along with increased coagulant properties. Another group also reported enhanced TF expression in A431 cervical cancer cells induced to EMT through EGFR activation or E-cadherin blockade (Milsom et al., 2008). Further strengthening the link between EMT programs and coagulation, we reported that silencing the EMT transcription factor ZEB1 inhibited both EMT-associated TF expression and coagulant activity. EMT-positive cells also exhibited a higher survival/persistence in lungs of mice colonized 24 hrs after intravenous injection, which was diminished by silencing of TF or ZEB1. Conversely, triggering *de novo* expression of Snail in MDA-MB-468 breast tumor cells was shown to increase TF, coagulant properties and early metastasis in mice. Tumor cells that persisted and stopped in the lungs were shown to be surrounded by fibrin and platelets. Supportively, using three-dimensional visualization of direct infusion of fluorescence labeled antibody to observe the interaction of tumor cells with platelets and fibrin(ogen) in isolated lung preparations, Im and co-workers also observed that tumor cells arrested in the pulmonary vasculature were associated with a clot composed of both platelets and fibrin(ogen) (Im et al., 2004). Thus, EMT-driven local coagulation around EMT-positive CTCs could thus generate a fibrin/platelet rich microclot around these CTCs. This fibrin-rich network could constitute a protective shield enhancing CTC survival by physically protecting the CTCs against shear stress or NK-mediated clearance, or by providing an anchoring matrix that would minimize anoikis.

Accordingly, a mechanism involving a protective role of fibrin and/or platelets in NK-induced cytotoxicity against tumor cells has been proposed based on *in vitro* observations and on preclinical experiments in mice using a variety of mouse and human carcinoma cell lines (Palumbo et al., 2000, 2005; Stegner et al., 2014; Lou et al., 2015; Leblanc and Peyruchaud, 2016; Li et al., 2016). Although platelet binding to CTCs may involve direct interactions, fibrin has also been shown to enhance adherence between platelets and tumor cells and to inhibit cytotoxicity against tumor cells (Biggerstaff et al., 1999, 2008). Palumbo et al. further showed that fibrin(ogen)-dependent and platelet-dependent evasion of NK cell-mediated clearance of early micro-metastases rely on TF expression by tumor cells, although they clearly demonstrated that NK-independent mechanisms are also involved in TF-mediated metastasis (Palumbo et al., 2007). Considering our findings linking TF expression, coagulation and EMT with these observations, it is conceivable that EMT-shifted CTCs may display enhanced potential to resist immune clearance.

Along these lines and providing clinical relevance for the involvement of an EMT-driven local activation of the coagulation cascade around the EMT-shifted phenotypes, our group identified a subpopulation of CTCs expressing vimentin and TF in the blood of metastatic breast cancer patients (Bourcy et al., 2016).

Formation of Specific Protective Cytoskeletal Structures (Microtentacles)

Another mechanism conveyed by EMT that may facilitate resistance to anoikis and the initial colonization of distant organs involves the formation of particular cytoskeletal structures (Matrone et al., 2010). Thus, it has been proposed that free-floating CTCs display cytoskeletal reorganization compared with tumor cells attached to a matrix, which help them resist to shear stress and anoikis. This mechanism involves the formation of so-

called microtentacles. These structures are built of Glu-tubulin (generating more stable detyrosinated microtubules) and are enhanced by vimentin, one of the most best-known markers of EMT (Whipple et al., 2008). Further implicating EMT in the process, Snail and Twist were also shown to regulate these structures in tumor cells (Whipple et al., 2010). Additionally, microtentacles have been reported to infiltrate the junctions between endothelial cells, suggesting that they may also facilitate early colonization by enhancing the reattachment of CTCs to the vascular endothelium (Whipple et al., 2010).

Immune Escape

The expression of programmed death-ligand 1 (PD-L1) at the surface of tumor cells is considered to play a pivotal role in the ability of tumor cells to escape elimination by the immune system. Indeed, PD-L1 and its programmed death receptor (PD-1) constitute a physiological immune checkpoint system. Thus, PD-L1 is expressed on antigen-presenting cells and PD-1 is expressed at the surface of activated T-cells. Upon binding of the ligand to the receptor, a strong proliferative inhibitory signal is sent to the T-cells. By expressing PD-L1, tumor cells have been shown to hijack this recognition mechanism to escape elimination by the immune system. Recently, PD-L1 has been detected on the surface of CTCs of breast cancer patients (Mazel et al., 2015), non small cell lung cancer patients (Nicolazzo et al., 2016), or bladder cancer patients (Anantharaman et al., 2016) and has been proposed as a mechanism enhancing CTC immune escape and survival (Wang et al., 2016; Alix-Panabieres et al., 2017). Independent studies have revealed that EMT pathways, such as the miR200/ZEB1 axis or the EMT-induced PI3K/Akt pathway, regulate PD-L1 in vitro (Chen et al., 2014; Alsuliman et al., 2015). An association between PD-L1 expression and EMT markers has also been observed in breast cancer or head and neck squamous cell carcinoma (Alsuliman et al., 2015; Ock et al., 2016). It is thus conceivable that CTCs with an EMT-shifted phenotype would be more efficient in hijacking this PD-1/PD-L1 immune escape system.

Although it has not been specifically examined in CTCs, it is interesting to note that other molecular mechanisms may be used by EMT-shifted cells to resist immune attack (Akalay et al., 2013; Tripathi et al., 2016). For instance, a depleted repertoire of HLA class I-bound peptides has been reported in mesenchymally-shifted non small cell lung carcinoma cells, thereby increasing their resistance to cytotoxic T lymphocytes (Tripathi et al., 2016).

Formation of Clusters of CTCs

The ability of CTCs to form and/or travel as homotypic or heterotypic (together with platelets, blood cells, or normal cells from the primary site such as fibroblasts and/or endothelial cells, ...) clusters has also been proposed as a mechanism enhancing their metastatic competence (Duda et al., 2010; Aceto et al., 2015; Cheung et al., 2016; Pothula et al., 2016), although the prevalence of hybrid E/M phenotypes in these structures is unclear. Such aggregates could indeed enhance physical protection of CTCs during their translocation in the circulation but also facilitate the attachment of CTCs to blood vessels and niche formation. Although much less prevalent than isolated CTCs, CTC clusters have been suggested to harbor high metastatic potential (Aceto et al., 2015). Accordingly, their presence has been associated with a poor

prognosis in several cancer types including breast, lung, and prostate cancers (Hou et al., 2012; Aceto et al., 2014; Paoletti et al., 2015; Wang et al., 2017a).

An early report by Glinsky and co-workers (Glinsky et al., 2003) showed in vitro, ex vivo, and in vivo that metastatic breast and prostate carcinoma cells form multicellular homotypic aggregates at the sites of their primary attachment to the endothelium. Alternatively, a study using fluorescently tagged tumor cell xenograft models, suggests that CTC clusters do not form within the circulation but are derived from grouping tumor cells that enter the circulation together (Aceto et al., 2014). These authors also revealed that ex vivo formed clusters are highly metastatic when injected intravenously in mice and that their metastatic potential relies on the expression of the cell-cell adhesion molecule plakoglobin. Plakoglobin was accordingly observed by these authors in CTC clusters in metastatic breast cancer patients (Aceto et al., 2014). Although the authors report that cell-cell contacts are present in these clusters, the EMT status in these entities is still underexplored (Aceto et al., 2015).

In a model of cell tracing in a MMTV-PyMT mouse background, Cheung et al. (2016) also observed polyclonal tumor cell clusters at different stages of metastasis including circulating tumor cell clusters. They further reported that cells in the clusters frequently expressed the epithelial marker keratin 14 and further demonstrated a functional role of K14 expression in metastasis. Interestingly, transcriptomic analyses revealed that the K14+ population not only expressed epithelial markers (several cell-cell adhesion molecules) but was also enriched for EMT/stemness markers such as Tenascin C or Jagged1 (Cheung et al., 2016). Along the same lines, an in vitro study has shown that the Notch-Jagged1 signaling pathway is involved in the formation of clusters exhibiting a hybrid E/M phenotype (Boareto et al., 2016).

Directly supporting the idea that circulating tumor emboli are made of cells expressing hybrid E/M phenotypes, the study of Yu et al. (2013) identified cells expressing both epithelial (such as EpCAM or cytokeratins) and mesenchymal markers (including fibronectin, N-cadherin or PAI-1) in isolated CTCs but also in CTC clusters from breast cancer patients. Vimentin expression has also been observed in circulating tumor microemboli detected in lung cancer patients (Hou et al., 2011). Because, as we discussed above, EMT contributes through diverse mechanisms to enhance CTC survival (activation of survival pathway, microtentacles, coagulation), the presence of EMT-shifted CTCs in clusters combined with the survival advantage of cell-cell interactions, would very likely overall enhance the survival of the clustered tumor cells (Aceto et al., 2015). Also, the possibility that activation of the coagulation cascade may contribute to the formation or the survival of the clusters cannot be excluded. Supportively, CTC clusters identified in breast cancer patients showed an abundance of attached platelets (Yu et al., 2013).

EMT Supports Metastatic niche Formation

Regardless of the suspected involvement of a MET in the final metastatic outgrowth, data suggest that CTCs expressing mesenchymal and cancer stem cell attributes harbor enhanced competence to accomplish the very early colonization steps and the formation of metastatic niches. CTC homing in colonized organs (thus becoming disseminated tumor cells, DTCs) involves interactions with the endothelium and the establishment of a favorable microenvironment, a metastatic niche, that will sustain their

growth after a possible period of dormancy. The molecular and cellular entities implicated, and the timing of metastatic niche formation, are still poorly understood but are being characterized. Although niche formation is likely to implicate a repertoire of cellular and molecular actors varying upon the context (Descot and Oskarsson, 2013), it is today clear that a particular matrix is created in the niche and that host cells, including immune cells and other stromal cells (activated fibroblasts, mesenchymal stem cells, endothelial cells, . . .) are recruited and are essential to the formation of the niche (Ombrato and Malanchi, 2014; Plaks et al., 2015). Interactions between the CTCs/DTCs and the niche microenvironment are determinant for the survival and the adaptation of the CTCs.

Thus, the EMT-associated cancer stem cell attributes and activation of the cell surviving mechanisms listed above are likely to cooperate to sustain self-renewal, survival, possible dormancy and therapeutic resistance of DTCs. Adding to this, some of these mechanisms may also directly contribute to the establishment of the niche and the recruitment of host cells. Therefore, if coagulation is suspected to protect CTCs during their travel in the bloodstream and during early hours of colonization, published literature also implicates coagulation in early phases of niche formation (Palumbo and Degen, 2007; Palumbo, 2008; Degen and Palumbo, 2012; Gil-Bernabe et al., 2013). Thus, both fibrin(ogen) and platelets may guide the formation of early metastatic niche by regulating CTC interactions with the endothelial wall, and platelets were also shown to enhance the recruitment of inflammatory cells into the niche (Im et al., 2004; Labelle and Hynes, 2012; Labelle et al., 2014; Lou et al., 2015; Leblanc and Peyruchaud, 2016; Tesfamariam, 2016). Importantly, TF was shown to enhance tumor cell survival after arrest in the lung during experimental lung metastasis and to stimulate metastatic niche formation by recruiting macrophages (Gil-Bernabe et al., 2012). Although the studies mentioned above have not directly examined a potential contribution of EMT, it is conceivable that EMT, by enhancing TF expression and the formation of microclots around CTCs, could directly modulate these processes.

Directly evidencing the importance of specific mesenchymal attributes of MICs in the establishment of the metastatic niche, an EMT axis modulated by the Axl receptor tyrosine kinase (RTK), has been shown to play a key role in the early establishment of metastatic niches by activating fibroblasts in the colonized organs as a result of thrombospondin 2 secretion (Ombrato and Malanchi, 2014; Del Pozo Martin et al., 2015). The authors demonstrated that the cancer cell acquired Axl-driven mesenchymal features facilitate fibroblast activation and the first phase of a metastatic niche formation. They then identified a second phase of metastatic colonization in which the activated stroma modulates the EMT of cancer cells toward a more epithelial state with loss of Axl and EMT markers expression. Additionally, using a model of MMTV-Her2 mice, Aguirre-Ghiso's group showed that HER2 + DTCs activate a Wnt-dependent EMT dissemination program without the complete loss of an epithelial phenotype. They further reported that these early disseminated cancer cells expressing Twist1 and a low level of E-cadherin are capable of forming metastasis after a dormancy phase (Harper et al., 2016). In agreement with other studies (Ocana et al., 2012; Tsai et al., 2012), these findings are consistent with the concept that mesenchymal attributes of hybrid MIC phenotypes are required for metastatic niche initiation and an eventual dormancy phase while a dynamic EMT inhibition (MET) at the secondary site occurs

within the second phase of metastatic colonization (Bednarz-Knoll et al., 2012; Brabletz, 2012; Tsai and Yang, 2013; Ombrato and Malanchi, 2014).

In line with these experimental observations, few reports have analyzed EMT markers in DTC from the bone marrow of cancer patients to examine their potential hybrid phenotype. So far, data have been essentially collected from cell lines established from DTCs isolated from bone marrow of cancer patients demonstrating the co-existence of epithelial (keratins, EpCAM) and mesenchymal/stem cell markers (vimentin, PAI-1, Twist, CD-44) (Willipinski-Stapelfeldt et al., 2005; Balic et al., 2006; Watson et al., 2007; Bartkowiak et al., 2009).

Functional Assays Reveal that MICs are Present in the Blood of Cancer Patients

The data reported so far thus support the idea that CTCs displaying hybrid phenotypes with mesenchymal and stem cell attributes would be those with enhanced metastatic competence. Ultimately, demonstrating whether MICs are present in CTCs of cancer patients relies on the development of assays allowing examination of the functional metastatic competence of CTCs (Alix-Panabieres et al., 2016). Thus, several *in vitro* cultures and assays have been developed to examine viability, proliferative, or invasive properties of CTCs isolated from cancer patients (Gao et al., 2014; Martin et al., 2014; Khoo et al., 2015, 2016; Alix-Panabieres et al., 2016). Nevertheless, the most obvious assay with which to examine the tumorigenic and metastatic competence of CTCs is the CTC-derived xenograft assay (CDX), consisting in injecting different subpopulations of CTCs isolated from cancer patients in immunodeficient mice (Hodgkinson et al., 2014; Yu et al., 2014; Cayrefourcq et al., 2015; Alix-Panabieres et al., 2016). Some authors also focused on examining CTC metastatic competence after intravenous injection of CTCs. Another variation consists in culturing and expanding the CTCs *in vitro* before injection in the animal. Using such *in vivo* assays, the presence of MICs within the CTC population of different types of cancers has been demonstrated (Rossi et al., 2014; Sullivan et al., 2014; Alix-Panabieres et al., 2016). Although further investigations and more extensive phenotyping are required, few studies more particularly support a potential relationship between hybrid E/M CTC phenotypes and metastatic competence. Thus, Zhang et al. established cell lines from breast cancer-derived, EpCAM-negative CTCs that expressed hybrid phenotypes with the expression of epithelial, mesenchymal and cancer stem cell markers (cytokeratins 8 and 18, vimentin, CD44) and that displayed metastatic competence after tail-vein or intracardiac injection (Zhang et al., 2013). They further established a molecular signature (HER2+/EGFR+/HPSE+/Notch1+) that accompanied enhanced metastatic competency in the brain. Injecting CTCs isolated from patients with metastatic breast cancer into the femur of immunocompromised mice, Baccelli et al. also demonstrated the existence of MICs that gave rise to bone, lung, and liver metastases. These MICs were found to express EpCAM, CD44, CD47, and c-MET (HGF receptor) (Baccelli et al., 2013). Additionally, the authors reported that the levels of CD44+/c-MET+/CD47 + CTCs, but not bulk CTCs, correlated with lower overall survival and increased number of metastatic sites.

Concluding Remarks

CTCs with hybrid epithelial/mesenchymal phenotypes and expressing stem cell markers are present in the blood of cancer patients harboring epithelial tumors. The detection of mesenchymally-shifted CTCs has further been associated with poor clinical parameters in several studies, stimulating their more systematic detection for predictive/prognostic perspectives. Adding to this, literature accumulates that functionally implicates EMT in the biology of CTCs and in the early phases of the metastatic spread. EMT may thus contribute to the liberation of CTCs into the bloodstream and provide CTCs with enhanced survival properties and increased ability to initiate metastatic niches. It has been suggested that the CTCs harboring mesenchymal attributes are those with an enhanced metastatic competence, so called MICs. Nevertheless, there is a repertoire of epithelial plasticity and a multiplicity of potentially metastable hybrid phenotypes. There is thus a clear need to identify specific molecular mediators of EMP that participate functionally in specific stages of the metastatic spread, so as to further refine therapeutic strategies.

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