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Review

En Route to Metastasis: Circulating Tumor Cell Clusters and Epithelial-to-Mesenchymal Transition

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Blood-borne metastasis accounts for the vast majority of cancer-related deaths and it is fueled by the generation of circulating tumor cells (CTCs) from a primary tumor deposit. Recent technological advances have made it possible to characterize human CTCs as they travel within the bloodstream. CTCs are found both as single cells and as clusters of cells held together by intercellular junctions. Although less prevalent, CTC clusters appear to have greater metastatic potential than single CTCs in the circulation. Both may exhibit shifts in expression of epithelial and mesenchymal markers, which may show dynamic changes during cancer progression. In this review we discuss recent insights into the biological properties of individual and clustered cancer cells in the circulation.

Cancer Cells in Circulation

Metastasis is the principal cause of death in patients diagnosed with invasive cancer. Cancer cells that leave the primary tumor site and are transported through the circulation to distant organs are referred to as CTCs. CTCs have been used as a noninvasive source of cancer cells for analysis of tumor genotypes (i.e., so-called liquid biopsy), but their detailed characterization also holds the key to understanding the biology of blood-borne metastasis. CTCs are extremely rare, even in patients with metastatic cancer (approximately one cancer cell among a billion normal blood cells), and their isolation is greatly subject to technological constraints [1,2]. Nonetheless, the number of CTCs may far exceed the number of established metastatic foci in an individual patient, arguing that only a minor fraction of CTCs are viable and capable of initiating a metastatic lesion. CTCs have been detected in the majority of epithelial cancers, such as those of the breast, prostate, lung, colon and pancreas, as well as in cancers that do not express markers of epithelial origin such as glioblastoma multiforme and melanoma [3–10]. Generally, CTCs are more prevalent in the blood of patients with established metastases, probably owing to a higher tumor burden, but they have also been detected in patients with localized cancers.

Our current understanding of how metastasis occurs is primarily derived from mouse models where the primary tumor site and size, its driving genetic alterations, and stage of metastatic lesions can be readily engineered and studied. However, mouse models alone cannot fully recapitulate the complexity of a human metastatic cancer. The emergence of increasingly advanced technologies to isolate CTCs from patient-derived blood specimens now provides the invaluable opportunity to test and validate our understanding of cancer metastasis. In this review we discuss recent findings in CTCs biology and focus on the role of cell–cell junctions, CTC clusters, and epithelial-to-mesenchymal transition (EMT) in the metastatic process.

Trends

New bioengineering technologies allow detailed characterization of circulating tumor cells (CTCs). These rare populations of cancer cells in transit within the blood circulation hold the key to understanding the process of human cancer metastasis.

While most CTCs are single cells, a small fraction travel as groups of cells. In mouse models, CTC clusters derive from individual tumor fragments and exhibit far greater metastatic potential than single CTCs.

Dynamic changes in epithelial and mesenchymal markers are evident within CTCs as patients initially respond and ultimately progress on cancer therapies, pointing to considerable plasticity of cell fates in cancer cells that evolve in response to treatment.

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 http://dx.doi.org/10.1016/j.trecan.2015.07.006

Early Insights from Cancer Histopathology

The search for factors that regulate cancer metastasis is traced to Paget, who in 1889 analyzed postmortem tissue from women who had died of cancer, and noticed the high frequency of metastases to the ovaries and bone. Paget proposed that the apparently restricted organ distribution of metastases is not a matter of chance, and that metastases develop only when particular tumor cells are compatible with specific organs [11]. These observations were the foundation of the 'seed versus soil' debate, contradicting the prevailing theory of Virchow, that metastasis could be simply explained by the arrest of tumor cell emboli in the vasculature [12]. More than a century later, these two models continue to frame the field as we strive to identify molecular mechanisms driving metastasis in different types of cancer.

Before the advent of CTC isolation technologies, important correlates of tumor invasion were established *in vitro* and by histopathological measurements. These include invasion of cancer cells through the basement membrane [13], their growth in semisolid agarose [14], and in 3D culture models admixed with stroma [15,16]. The role of EMT in metastasis was first established in mouse tumor models. EMT is a physiological switch in cell fate, whereby immobile epithelial cells acquire migratory properties of mesenchymal cells, and it is essential for the creation of neural crest lineages, heart valves, and other differentiated structures during embryogenesis [17]. Aberrant activation of EMT, through expression of master transcriptional regulators (e.g., Twist, Snail) or intercellular signaling pathways (e.g., transforming growth factor β, TGF-β) have been described [18,19]. In addition, EMT-inducing transcription factors have been implicated in malignant transformation, tumor formation, and escape from oncogene-induced senescence in mice [20–23], and their overexpression promotes intravasation of cancer cells into the circulatory system [24]. Upon arriving at distant sites, the presentation by tumor cells of integrin β1-containing filopodium-like protrusions (FLPs), another EMT-driven phenomenon, may facilitate their interaction with the extracellular matrix and the extravasation process [25]. However, established metastases are primarily epithelial in appearance, suggesting the reverse switch in cell fate, mesenchymal-to-epithelial transformation (MET), is required for cancer cells to regain their full proliferative phenotype, which is suppressed by mesenchymal transformation [26]. Altogether, the current predominant understanding of how metastasis occurs is based on experimental preclinical models that led to the notion that single cancer cells undergoing EMT can actively intravasate in the bloodstream and extravasate at distant sites, where they revert to an epithelial fate and initiate metastatic lesions [26–28].

The EMT phenomenon has been difficult to validate using standard histopathological analysis of human primary and metastatic cancers owing to the presence of stromal cells, which are strongly positive for mesenchymal markers and thus obscure the detection of epithelial tumor cells transitioning to a mesenchymal state [29–31]. In histopathological sections, most epithelial cancers seem to display the hallmarks of collective invasion into surrounding tissues, including intact cell–cell junctions, expression of E-cadherin as well as other cadherins, and expression of hemophilic cell–cell adhesion receptors [31–34]. So-called disseminated tumor cells (DTCs) in the bone marrow of patients with epithelial cancers are almost exclusively of epithelial phenotype, either as single cells or as micrometastatic clusters [35–37]. Demonstrating the contribution of EMT to cancer metastasis thus requires the ability to isolate cancer cells in the act of invasion and as they transit in the bloodstream.

Isolation of Cancer Cells in Circulation

CTCs were first reported in 1869 in a postmortem blood analysis [38]. In recent years several technologies for CTC isolation have been developed, with the goal to purify this rare population of cells in a viable state from the vast number of surrounding blood cells. This goal has proven to be extraordinarily challenging owing to the low number of CTCs and their variable expression of cancer-associated markers [1,2]. Multiple technologies for CTC isolation have emerged in

parallel, each focused on differential characteristics that distinguish cancer cells from normal blood cells, including the expression of cell surface markers, and physical or functional properties.

Tumor-Antigen-Dependent Capture of CTCs

Most cancer cells in circulation that originate from an epithelial tumor are thought to express the EpCAM (epithelial cell adhesion molecule) antigen on their cell surface. Among the numerous EpCAM-based CTC isolation technologies, the CellSearch® system is the most frequently used and currently the only one that is approved by the US FDA. For this reason, CellSearch® is considered as a benchmark for all other CTC-detection methods [39]. Using this assay, elevated CTC counts have been associated with a decreased progression-free survival and overall survival in patients with breast, prostate, and colorectal cancers [7,40–42]. More recent and more sensitive microfluidic cell isolation platforms include the micropost-based CTC chip and the herringbone (HB)-based CTC chip [4,6,43,44]. These specially designed devices can be functionalized not only with EpCAM but also with antibody cocktails that recognize additional cancer associated antigens, such as epithelial growth factor receptor (EGFR) and HER2 (EGFR2/ERBB2) [3]. Multiple other technologies for CTC isolation are reviewed elsewhere [1,2]. An intriguing alternative to *ex vivo* isolation of CTCs was developed by GILUPI GmbH (CellCollector™) to capture CTCs *in vivo*, using an indwelling anti-EpCAM-coated wire inserted into a vein [45]. While more invasive than analysis of routine blood specimens, this approach may interrogate larger blood volume for the presence of rare cells. However, a major caveat of all these methods is the variability in cell surface expression of markers by diverse and heterogeneous cancer cells, including the loss of EpCAM expression in tumor cells that undergo EMT.

Tumor-Antigen-Independent Purification of CTCs

A strategy to circumvent the reliance on common CTC markers is to deplete all normal blood cells from a blood sample, leaving behind an enriched population of untagged CTCs. The recently developed CTC-iChip achieves initial hydrodynamic separation of nucleated cells from smaller blood components (i.e., platelets and red blood cells, RBCs), followed by inertial focusing of all nucleated cells (i.e., CTCs and white blood cells, WBCs) into a single file along the microfluidic channel. This allows highly efficient immunomagnetic depletion of antibody-tagged WBCs, thereby purifying untagged and unmanipulated CTCs in solution [46]. This approach has the advantage of efficiently isolating CTCs independently of presumed tumor-specific surface markers. However, a limitation of the CTC-iChip is that its flow kinetics are optimized for the isolation of individual cells or small CTC clusters (2–4 cells). Larger CTC clusters or tumor microemboli may not enter the device. To address this challenge, another microfluidic device specifically designed to capture CTC clusters was developed [47]. This cluster chip relies on the strength of cell–cell junctions within a CTC cluster to wedge grouped cells in specially designed microfluidic pores. Two triangular pillars form a gradually narrowing channel that funnels the cells into an opening where the apical edge of a third pillar is positioned to bifurcate the laminar flow. Unprocessed whole blood is flowed through the device, and captured CTC clusters are released by reversal of flow. Compared to filter-based technologies, such as the ISET®, ScreenCell®, Parsortix, and JETTA™ that also capture CTCs on the basis of cell size [48–50], the cluster chip operates at sub-physiological flow rates, which has the advantage of preserving structural integrity and viability of CTC clusters, and preventing these highly deformable cell groupings from squeezing through small pores under higher flow pressures [47]. Other size-based microfluidic isolation technologies have been reported [51,52], and CTC clusters are also visualized by direct precipitation of all blood cells onto specially prepared slides, followed by high-speed microscopic scanning [53].

Studies of CTC Clusters in Circulation

While single cancer cells in circulation represent the vast majority of CTCs, clusters of circulating tumor cells were first predicted and then observed several decades ago in patients with cancer

[12,54,55]. Intravascular tumor microemboli, representing multicellular epithelial tumor cells, are often detected as an incidental finding during autopsy of cancer patients [56]. The most common malignancies associated with development of tumor emboli are mucin-secreting adenocarcinomas originating in the breast, colon, lung, or stomach, as well as hepatocellular carcinoma, prostate cancer, choriocarcinoma, and renal cell carcinoma [57–59]. Although clinical manifestations of tumor embolism are often indistinguishable from the more common thromboembolism, it is generally accepted that the presence of tumor microemboli in the circulation is associated with poor clinical outcome [31,60].

Consistent with the detection of tumor emboli in clinical specimens, tumor cell clusters in the blood have been observed using various technologies in cancers of the breast, pancreas, kidney, colon, lung, and melanoma [3,5,10,44,61–64]. The presence of CTC clusters in the blood has also been associated with a poor prognosis in patients with lung, breast, or prostate cancers [65–67]. In a series of mouse modeling experiments using differentially tagged primary tumor cells, it was recently shown that CTC clusters are not derived from intravascular aggregation of single CTCs or from the progeny of a single primary tumor cell that proliferates in the vascular space. Instead, CTC clusters are oligoclonal in origin, derived from groupings of primary tumor cells that together enter the circulation [66]. These conclusions were reached from two different mouse models. In the first model, GFP- or mCherry-tagged breast cancer cells were coinjected into the mouse mammary gland to form a multicolor tumor. Virtually all tumor-derived CTC clusters were found to be multicolor and they gave rise to multicolor metastases. This indicated that CTC clusters are oligoclonal in origin and are not derived from the progeny of a single tumor cell proliferating inside a blood vessel. In the second model, GFP-tagged breast cancer cells were injected into the right mammary gland of mice, while mCherry-tagged breast cancer cells were simultaneously injected into the left mammary gland. In this case, tumor-derived CTC clusters as well as metastatic foci predominantly consisted of tumor cells of a single color (either GFP or mCherry). This observation excluded intravascular aggregation of individual CTCs to generate CTC clusters. Together, these modeling experiments indicate that CTC clusters arise from the release of grouped cells from a single tumor deposit into the circulation. By comparing the relative frequency of single CTCs versus CTC clusters in circulation with that of lung metastases derived from these color-coded precursors, CTC clusters were estimated to be up to 50-fold more metastasis-competent than single CTCs.

Cell–Cell Junctions in CTC Clusters

At the level of single cell resolution RNA sequencing, single CTCs and CTC clusters isolated from the blood of an individual with metastatic cancer are highly similar. However, among the few differences are high-level expression of the cell–cell junction marker plakoglobin, increased ~200-fold in CTC clusters from women with metastatic breast cancer [66]. Plakoglobin is a crucial intracellular component of both adherence junctions and desmosomes [68,69], with variable properties in different tumor models [70–72]. In an analysis of publicly-available datasets, including a total of more than 1900 breast cancer patients, high expression of plakoglobin in the primary tumor correlated with decreased metastasis-free survival [66]. Plakoglobin is expressed in a heterogeneous fashion in primary breast tumors, with areas of high expression interspersed with areas where plakoglobin is not detectable. Thus, plakoglobin expression might demarcate areas of more tightly tethered tumor cells that may be precursors of CTC clusters. It is not clear, however, whether CTC clusters passively enter the bloodstream through compromised tumor vasculature or whether they actively traverse the endothelial cell barrier as observed in collective cell migration phenomena [33,73]. Interestingly, *in vitro*, plakoglobin knockdown efficiently disaggregates clusters of neoplastic cells, while it has far less impact on non-transformed cells, suggesting that multiple normal cell–cell junction components may be reduced in tumor cells, thereby increasing their dependence on plakoglobin for intercellular junctions [66]. Beyond plakoglobin, other cell–cell junction markers are also overexpressed in CTC clusters

compared to single CTCs [66], but much remains to be learned about how intercellular junctions within CTC clusters may differ from those of primary tumor cells, as well as of normal epithelia. Such information may bear on whether cancer cell clustering in the blood confers resistance to the cells against apoptosis [65], but at the same time it may open the door to new therapeutic targets directed against cell–cell junctions and associated survival pathways. By analogy with human pluripotent stem cells (hPSC), self-renewal, proliferation, and differentiation require cell–cell adhesion regulated by E-cadherin and several other cell adhesion molecules (CAMs) including cadherins, Ig-superfamily CAMs, integrins, and heparin sulfate proteoglycans [74]. In a similar fashion, cell–cell junctions may play a significant role in the ability of cancer cells to survive in the bloodstream and achieve successful metastatic colonization.

Fate and Circulation Time of CTC Clusters

How do CTC clusters navigate through the narrow capillary beds of distant organs and survive the shear forces in the blood circulation? For instance, a CTC cluster from a metastatic prostate cancer bone lesion would have to travel first through pulmonary capillaries and then through capillaries in the hand and fingers before being collected in a blood sample drawn from the arm. *In vitro* reconstitution experiments suggest that grouped cancer cells exhibit remarkable deformability as they pass single file through microfluidic channels that are matched to physiological diameters and pressure gradients (unpublished). In addition, while some capillary beds may prove impassable for large CTC clusters, others may contain larger arterio-venous channels that may allow their passage. The circulation time for CTCs in patients with breast cancer has been modeled as being between 1 and 2.4 h [75], and in patients with localized prostate cancer who have CTCs preoperatively, most, but not all, have no detectable CTCs 24 h after surgical resection of the tumor [4]. Using *in vivo* flow cytometry applied to a mouse model with labeled CTCs, the half-life of injected CTC clusters was determined to be approximately 10 min, versus 30 min for single CTCs. The shorter half-life of CTC clusters is consistent with their entrapment within small capillaries of distant organs, but it is considerably longer than a single pass through the circulatory system, suggesting that at least a subset of CTC clusters persist through multiple capillary barriers. It is also possible that a far larger number of clusters are released by tumor deposits and quickly trapped in lung capillaries, thereby being invisible to most blood sampling methods. Further studies, using intravital imaging approaches, will be necessary to elucidate the mechanisms of CTC cluster dissemination and clearance from the circulation.

Survival Advantage of CTC Clusters

Generally, epithelial cells that have lost adhesion-dependent survival signals by transitioning from a primary tumor into the bloodstream undergo anoikis. Several different mechanisms may serve to mitigate this cell death pathway. During EMT, individual cancer cells may evolve toward a mesenchymal fate in which adherence-independent survival signals compensate for the loss of intercellular junctions and basement membrane. Alternatively, CTC clusters may preserve some of the crucial cell junctions and may be sufficient to suppress anoikis. Beyond survival in the bloodstream, CTC clusters may also provide intercellular signals that may facilitate the outgrowth of cancer cells once lodged in distant organs. Indeed, mouse tumor modeling experiments were crucial to observe reduced apoptosis and more rapid proliferation of tagged cancer cells disseminated to the lung as clusters rather than single cells [66]. In addition to cancer cells, virtually all CTC clusters are coated with platelets, and some may include adherent WBCs or even tumor-derived fibroblasts or endothelial cells [44,47,76,77]. The fact that CTC clusters in some cases be 'seeds bringing their own soil' may contribute to further help their growth at a distant site.

EMT in Circulating Tumor Cells

The routine use of epithelial markers to define CTCs has made it difficult to demonstrate expression of mesenchymal markers, many of which are present in normal blood cells. Recently,

Key Figure

Steps Along the Metastatic Cascade

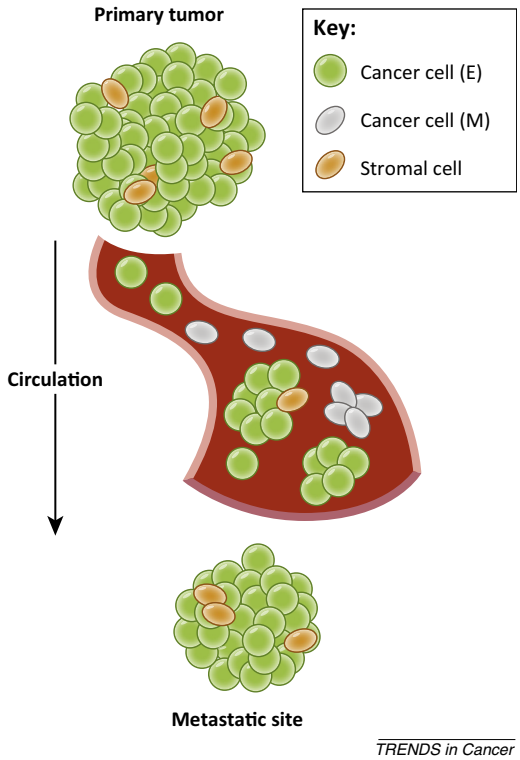

Figure 1. Cancer cells within the primary tumor and metastatic deposits are almost exclusively found in an epithelial (E) state and admixed with stromal cells. In circulation, cancer cells adopt different mechanisms that favor their survival such as clustering, interaction with stromal cells, and the acquisition of a mesenchymal (M) phenotype. These mechanisms of cancer dissemination warrant further investigation to reveal and ultimately target an Achilles' heel within the metastatic process.

the use of antibody cocktails against multiple cell surface epitopes to isolate CTCs, combined with specific and quantitative scoring for expression of epithelial versus mesenchymal transcripts using RNA *in situ* hybridization (RNA-ISH), made it possible to definitively demonstrate EMT within individual CTCs [3]. Remarkably, however, expression of mesenchymal transcripts by CTCs was not constant in cells from cancers from an individual patient. Instead, dramatic shifts in epithelial versus mesenchymal composition was evident as a function of response or progression to sequential treatment regimens. In CTCs isolated from an individual patient, the fraction of mesenchymal-predominant cells rose as the cancer became resistant to a therapeutic regimen, only to drop precipitously as a new effective treatment was introduced, leading to tumor shrinkage [3]. These observations suggest that EMT features in CTCs may not solely reflect their intrinsic invasiveness but they may also be modulated by survival and drug-resistance pathways initiated by therapeutic interventions.

Consistent with this model, CTC clusters in patients receiving anticancer therapies express as many if not more mesenchymal transcripts than single CTCs. In these clusters, persistent expression of epithelial junction components is required for stability of the tumor cell cluster itself, while partial acquisition of mesenchymal cell fate may further enhance survival signals. Recent

modeling studies have suggested that the core decision network regulating cancer EMT may allow transition into a hybrid epithelial/mesenchymal (E/M) or partial EMT phenotype [78,79], exhibiting a combination of epithelial cell–cell adhesion and mesenchymal motility traits, which together may enhance collective cell migration as seen in wound healing, tissue morphogenesis, and some cancer models [33,73,80,81]. In this context, CTC clusters could be highly effective metastatic effectors, combining the advantages of intercellular interactions with cellular deformability and motility. Further studies will be needed to specifically address these models in clinically-relevant settings.

Of note, RNA sequencing of single CTCs isolated from patients with diverse cancers reveals considerable plasticity in their expression profiles: whereas mesenchymal markers are detected in breast cancer CTCs [66], they are rare in prostate cancer CTCs (unpublished). Both human and mouse pancreatic CTCs show preservation of some epithelial markers but striking acquisition of stroma-associated extracellular matrix (ECM) transcripts [82].

Concluding Remarks

Taken together, the emerging molecular characterization of CTCs, a previously inaccessible transient population of cancer cells at a crucial juncture in metastasis, is likely to yield new and profound insights into the mechanism by which human cancer disseminates to distant organs (Figure 1, Key Figure). The combination of manipulable mouse models and direct analysis of patient-derived specimens is essential to ascertain the full complexity of the metastatic process, including both individual cells with invasive EMT phenotypes and CTC clusters or microemboli (see Outstanding Questions). In addition, the predominant mechanisms of tumor dissemination may vary in different types of cancer, many of which display characteristic sites of metastasis (e.g., to lung vs bone, liver, or brain). These predilections may reflect inherent properties of tumor cells, including their complement of cancer gene mutations and the effect of stroma-derived signals within the primary tumor. They may also indicate the expression of 'homing' receptors or cell surface molecules involved in tissue-specific tropism, as well as their dependence on growth signaling pathways that may predominate in some tissues more than others. In other cases, physical considerations, such as the integrity of the tumor vasculature, or its normal drainage pattern, may influence the properties and destination of cancer cells in the circulation. Ultimately, the goal of suppressing metastatic dissemination of cancer will require a detailed understanding of the key pathways that may be targeted therapeutically. Defining the unique characteristics acquired by cancer cells that survive transiently in the circulation is a crucial step in meeting this challenge.

Outstanding Questions

What are the mechanisms that cause the release of single versus clustered CTCs from a primary tumor?

Do single tumor cells undergoing EMT and tumor cells within CTC clusters contribute differentially to metastasis in various cancer types and at different stages of cancer progression?

What is the contribution of the stromal cells within CTC clusters to the metastatic process?

Do CTC clusters contain specialized populations of leukocytes?

Do single CTCs and CTC clusters express biomarkers/signaling molecules that determine tropism?

What are the biophysical properties of CTCs and the dynamic changes in cell–cell interactions that enable them to pass through the capillary beds?

Can CTC clusters be targeted pharmacologically?

Acknowledgments

We apologize to our colleagues whose work could not be cited owing to space constraints. We are grateful to all members of the laboratories of D.A.H. and S.M. for helpful discussions and for support from the National Institute of Biomedical Imaging and Bioengineering, National Cancer Institute, Howard Hughes Medical Institute, Breast Cancer Research Foundation, Stand Up to Cancer, National Foundation for Cancer Research, Susan G. Komen for the Cure, ESSCO Breast Cancer Fund, and the Massachusetts General Hospital (MGH)–Johnson & Johnson Center for Excellence in CTCs. N.A. is a fellow of the Human Frontier Science Program, the Swiss National Science Foundation, and the Swiss Foundation for Grants in Biology and Medicine.

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