REVIEW

A conduit to metastasis: circulating tumor cell biology

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Advances in the enrichment and analysis of rare cells from the bloodstream have allowed for detection and characterization of circulating tumor cells (CTCs) from patients with cancer. The analysis of CTCs has provided significant insight into the metastatic process. Studies on the biology of CTCs have begun to elucidate the molecular mechanisms of CTC generation, intravasation, survival, interactions with components of the blood, extravasation, and colonization of distant organs. Additionally, the study of CTCs has exposed dramatic intrapatient and interpatient heterogeneity and their evolution over time. In this review, we focus on the current knowledge of CTC biology and the potential clinical implications.

Advancements in the treatment of cancer and efforts to improve early detection over the past few decades have contributed to significant improvements in the survival of patients diagnosed with cancer. Newly developed treatments include novel targeted drugs against key oncogenic pathways (Robert et al. 2015) and immunotherapies (Brahmer et al. 2015). However, despite these new developments, metastatic disease remains incurable in the vast majority of cancer patients.

Metastasis is a complex, multistage process that requires the acquisition of diverse properties by cancer cells at precise times. Metastatic cells must invade and move from the primary tumor; access, survive, and then exit the bloodstream; colonize a distant tissue; and ultimately grow into a macroscopic metastatic lesion. Circulating tumor cells (CTCs) represent an intermediate stage of metastasis. While rare (estimated to be as low as one to 10 cells per 10 mL of blood), they are uniquely accessible through simple noninvasive blood sampling (i.e., phlebotomy). While some CTCs passively enter the bloodstream, CTCs derived from actively invading cells acquire key properties required for metastatic spread while still facing significant subsequent barriers to generate a metastatic lesion. CTCs can circulate as single cells or clusters of cells, with clusters appearing to have increased metastatic potential and a shorter half-life in the circulation (6–10 min for clusters vs. 25–30 min for single cells) (Aceto et al. 2014). Most CTCs die in the circulation, likely from a combination of physical stress, oxidative stress, anoikis, and the lack of growth factors and cytokines. Those CTCs that do survive either actively extravasate into the surrounding tissue or become lodged in a capillary bed. Once the cancer cells exit the bloodstream, they may begin to divide and colonize. However, more frequently, disseminated tumor cells spread throughout the parenchyma of the major organs and persist there, with only a small minority of these disseminated cells (estimated from experimental models to be 0.02%) generating a proliferating metastatic lesion (Hedley and Chambers 2009; Klein 2011; Gomis and Gawrzak 2017). Some of these disseminated cells enter a dormant state but appear to retain the ability to ultimately grow into a metastatic lesion. Other disseminated tumor cells may reach an equilibrium between cellular proliferation and cell death or elimination that prevents outgrowth of the lesion. The dormant cells remain clinically undetectable. Notably, in breast and prostate cancer, dormant cells can result in late relapses that occur years after the initial diagnosis and treatment. Dormancy appears to be maintained by a combination of cell-intrinsic mechanisms, microenvironmental factors, and immune surveillance (Aguirre-Ghiso 2007). Once outgrowth of disseminated disease occurs, secondary CTCs are generated.

In metastatic disease, CTCs are postulated to be derived from both the primary and metastatic lesions and, in the case when the primary has been resected, only from the metastatic lesions. Metachronous metastases are common in breast cancer, prostate cancer, colon cancer, and melanoma. Recent studies of independent metastatic sites within a single patient reveal that each site can

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evolve independently and acquire de novo mutations (Juric et al. 2015). Since primary tumor resections and single-site biopsy of metastatic lesions are unable to capture the global landscape of mutations across multiple sites and are prone to sampling bias, CTCs may provide a more global sampling of the entire population of invasive cancer cells. However, this concept still needs to be rigorously evaluated. CTC sampling is also amenable to multiple collections over time, allowing for detailed studies of the evolution of invasive cancer cells during the course of treatment. With this information, actionable mutations can be identified, and treatments can be tailored to the evolving cancer. The recent advances in technology for the enrichment and characterization of CTCs have provided new insight into the mechanisms of metastatic spread and offer the opportunity to identify potential targets that specifically inhibit metastasis. It has become clear that critical determinants of metastasis are the tumor cell's access to the bloodstream, survival in the blood, exit from the vasculature, and interaction with the distal tissue microenvironments. This review focuses on the current state of knowledge of CTC characterization and biology and the features of CTCs that lead to successful metastatic colonization.

Enrichment of CTCs

Technologies for the enrichment and isolation of CTCs have ranged from the simple to the sophisticated. Peripheral blood mononuclear cells can be enriched with chemical lysis of red blood cells, leaving the rare CTCs intermixed with mononuclear immune cells (Hensler et al. 2016). The benefit of this technique is that there is no selection bias; however, the large background of white blood cells (WBCs) prevents detailed analysis of CTCs. CTCs have also been isolated by physical characteristics, including deformability, density, and cell surface charge (Table 1; Liu et al. 2015; Mitchell et al. 2015; Shaw Bagnall et al. 2015). Compared with WBCs, epithelial cells tend to be larger, and numerous size-based filtration technologies have been developed to enrich for CTCs. However, size variability in CTCs is considerable and has significant overlap with WBCs. Thus, the challenge with these size-based selection technologies is ensuring the capture of the full range of CTCs.

The majority of CTC enrichment methods is based on immunoaffinity separation techniques. These techniques use high-affinity antibodies to cell surface markers. There are two approaches using this technique. The first uses cancer-specific markers to positively select for the CTCs. The most common marker used is EpCAM, a cell surface protein expressed on most epithelial cells—normal and neoplastic. EpCAM is one of the markers used in CellSearch, an FDA (Food and Drug Administration)-approved technology for the enrichment of CTCs. While CTC quantitation using this approach has been shown to be indicative of clinical outcome, the technology is limited by the a priori selection of a cell surface marker expressed on the cancer cell surface but not on other cells found in the circulation. Studies of CTCs have revealed significant heterogeneity, including the expression of cell surface markers. In particular, CTCs can exhibit features of epithelial-to-mesenchymal transition (EMT), including the loss of EpCAM (Yu et al. 2013). As a result, technologies based on positive selection are prone to enrich for a subpopulation of CTCs, while CTCs without expression of the chosen marker are lost. Current strategies combine antibodies directed against multiple cell surface

Table 1. *Techniques for CTC enrichment and analysis*

Basis for enrichment	Techniques	Selected references
Biophysical properties		
	Deformability	Shaw Bagnall et al. 2015
	Density	Liu et al. 2015
	Cell surface charge	Mitchell et al. 2015
	Size	Hou et al. 2013
Immunoaffinity		
Positive selection	EpCAM-based (CellCearch)	Allard et al. 2004
Negative selection	CD45, CD15, CD66b (iChip)	Ozkumur et al. 2013; Karabacak et al. 2014
Analysis	**Techniques**	**Selected references**
Enumeration	Immunofluorescence	Miyamoto et al. 2012; Tsai et al. 2016
Genomic analysis	Targeted DNA sequencing	De Luca et al. 2016
	Digital droplet PCR	Reid et al. 2015
Transcriptomic analysis	RNA in situ hybridization	Yu et al. 2013
	Single-cell RNA sequencing	Ting et al. 2014; Miyamoto et al. 2015
	Digital droplet PCR	Kalinich et al. 2017
Epigenetic analysis	Targeted bisulfite sequencing	Pixberg et al. 2017
Proteomic analysis	Mass cytometry	For review, see Spitzer and Nolan 2016
	Microfluidic Western blot	Sinkala et al. 2017
	Single-cell mass spectroscopy (under development)	For review, see Armbrecht and Dittrich 2017
Multimodal analysis	Glucose uptake, protein analysis, and mutational analysis	Zhang et al. 2015
	High-throughput imaging of unpurified cell preparations	Dago et al. 2014

markers to capture tumor cells expressing either epithelial or mesenchymal markers (Satelli et al. 2015). These antibody cocktails increase the proportion of CTCs enriched but still may miss specific subpopulations. In addition, each cancer type will require a different set of selection markers to optimally isolate CTCs.

Negative selection represents an alternative approach to the enrichment of CTCs that is not biased by the selection of potentially variably expressed markers on tumor cells. In this approach, blood components, including red blood cells, white blood cells, and platelets, are depleted from the sample. Red blood cells can be removed through size separation or red blood cell lysis. WBCs can be removed by high-affinity antibodies and immunomagnetic separation. Several highly specific antibodies for WBC markers have been developed for both human and mouse immune cells, including CD45, CD15, and CD66b (Ozkumur et al. 2013; Karabacak et al. 2014). Using this approach, CTCs are enriched in the final product through depletion of the normal blood components. This does not require a priori knowledge of CTC-specific cell surface markers and therefore captures a larger proportion of CTC heterogeneity. In addition, the development of cancer-specific enrichment strategies is not necessary. However, one of the challenges in the enrichment of these rare cells using immunomagnetic technology is the removal of the large number of WBCs needed to produce a high level of CTC enrichment (one to 10 CTCs admixed in upward of 50 million WBCs). To address this concern, our group has developed the CTC-iChip, a microfluidic device with a two-stage separation, which consists of a size-based removal of red blood cells followed by inertial focusing of the remaining cells into a single-file stream. Alignment of cells into a single microfluidic row allows for precise and controlled deflection of labeled cells (Karabacak et al. 2014). This device successfully enriches for CTCs in multiple cancer types (Miyamoto et al. 2015; Jordan et al. 2016). Key to the validation of the isolated CTCs is the determination of their neoplastic origin. Since the technique is unbiased in its inclusion of cells that are not WBCs, rare circulating epithelial cells and other nonimmune cells may be captured.

The enrichment of CTC clusters is an area of active research. With evidence suggesting that CTC clusters may have higher metastatic potential and exhibit distinct gene expression programs (Aceto et al. 2014), there is a need to develop technology that isolates the clusters and maintains their integrity. Some of the established enrichment techniques, including filtration and immunoaffinity, will also enrich for clusters, but how well the integrity of these fragile cellular conglomerates is maintained is not clear. Other technologies, including the CTC-iChip, are unable to isolate large intact clusters that are likely dispersed during processing, although small clusters can also be isolated. Some technologies, including flow cytometry-based techniques, may eliminate the cells of the clusters from the enriched product. This is an important consideration in the study of CTC biology and a caveat for studies based solely on single-cell CTCs. Future studies will need to address both single-cell CTCs and clusters. Microfluidic technology has been developed to specifically isolate large CTC clusters (Sarioglu et al. 2015; Au et al. 2017), and studies are ongoing with these techniques. There is a need for continued technological advances to enrich single CTCs and clusters of CTCs simultaneously and allow for the viable recovery and subsequent analysis.

Analysis of CTCs

Staining of CTCs

Once the CTCs have been enriched, numerous analytic techniques have been explored to study these rare cells (Table 1). Simple enumeration of the CTCs through staining with tumor-specific antibodies is an important clinical parameter in multiple cancers, including breast (Cristofanilli et al. 2004), prostate (de Bono et al. 2008), colon (Cohen et al. 2008), and lung (Krebs et al. 2011) cancer, where the pretreatment CTC enumeration predicts overall survival and/or progression-free survival. In these studies, CTCs were enriched using the FDA-approved CellSearch (Veridex LLC) technology, an epithelial marker-based method, and enumerated by staining with antibodies against cytokeratins. The presence of five or more CTCs per 7.5 mL of patient blood was associated with poor clinical prognosis. Recent studies have begun to explore the use of longitudinal change in the number of CTCs within an individual patient versus an absolute CTC cutoff, although the utility of this approach is still under investigation (Cheng et al. 2016). While CTC staining provides a valuable and quantitative measure of CTC burden, it is limited by the number of antibodies that can be used for visualizing the cells and the need for laborious viewer-dependent scoring and does not allow for comprehensive analysis of CTC function.

Genomic analysis of CTCs

Genomic analysis of CTCs provides clinically valuable information about the DNA mutational status of cancer and its evolution. As targeted therapies continue to be developed for specific mutations, accurate identification and monitoring for the emergence of de novo mutations during the metastatic process will be critical. For example, in lung cancer, the emergence of the *T790M* mutation in the *EGFR* gene is an important marker of resistance to first- and second-generation EGFR inhibitors. With the development of third-generation EGFR inhibitors with activity against the *T790M* mutation, detection of this mutation during the course of the disease can direct subsequent treatment (Janne et al. 2015; Sequist et al. 2015; Sundaresan et al. 2016). In breast cancer, similar mutational analysis of CTCs has been performed by next-generation sequencing and has revealed significant interpatient and intrapatient heterogeneity that can be monitored over time, including the emergence of activating *estrogen receptor* gene (*ESR1*) mutations (Yu et al. 2014; De Luca et al. 2016). The advantage of using CTCs for the monitoring of de novo mutational events is the

noninvasive collection of samples, the ability to easily acquire samples over time, and likely a decreased risk of sampling bias. In particular, in metastatic disease, CTC analysis allows for the analysis of a patient's tumor when biopsy of a metastatic lesion maybe too risky or unfeasible. However, there remain challenges to the deployment of these CTC technologies to clinical care, including the lack of a standard enrichment techniques, the few if any CTCs found in early stage disease, and the need for specialized technology to isolate and analyze DNA from limited cells. CTC analysis is likely to be complementary to current practices, including analysis of the primary tumor, although it may decrease the need for repeated biopsies of metastatic lesions. Increasingly, testing of circulating tumor DNA (ctDNA) in plasma has emerged as the easiest path for noninvasive tumor genotyping (Wan et al. 2017).

Gene expression analysis of CTCs

Transcriptome analysis of CTCs has contributed significantly to our understanding of the metastatic process. With the advancement of single-cell technologies, individual cells can be analyzed and compared with the primary or metastatic biopsies. CTC heterogeneity can be assessed within a single patient and compared with other patients over time. Transcriptome analysis using either targeted evaluation of a defined gene set or global single-cell RNA sequencing (RNA-seq) has been successfully used in isolated CTCs. For example, RNA-seq of single cells derived from a mouse model of pancreatic cancer identified noncanonical Wnt signaling and, specifically, Wnt2 as a gene expressed in CTCs that is important for metastatic spread (Yu et al. 2012). Similarly, in prostate cancer, single-cell RNA-seq from prostate cancer patients also identified the Wnt5a pathway as increased in patients treated with an androgen receptor (AR) antagonist and mediated an attenuated anti-proliferative response to the inhibitors (Miyamoto et al. 2015). More recently, digital PCR has also been used in the assessment of cancer-specific gene panels. In hepatocellular carcinoma (HCC), digital PCR analysis of a liver-specific RNA panel in CTCs provided orthogonal information to the α fetal protein (AFP) levels currently used to monitor high-risk patients (Kalinich et al. 2017). In addition to sequencing-based approaches, multicolor RNA in situ hybridization (ISH) can evaluate expression levels of multiple gene targets at the single-cell level in CTCs and has revealed remarkable heterogeneity (Yu et al. 2013).

DNA methylation in CTCs

DNA methylation profiles of CTCs in breast and prostate cancer revealed promoter methylation patterns for EMT-related genes that closely resembled epithelial cells but also demonstrated heterogeneity among CTCs (Pixberg et al. 2017). Targeted analysis of promoter methylation has been reported in breast cancer CTCs for *Sox17*, *BRMS1*, and *CST6*. For BRMS1 and CST6, promoter methylation was increased in metastatic patients versus patients with operable disease, potentially suggesting different biologic properties of CTCs derived from patients with metastatic versus localized disease (Chimonidou et al. 2011). Future studies are needed to evaluate the feasibility of reliable CTC detection in early disease to then assess global patterns of DNA methylation in CTCs at different stages of disease to give a more complete picture of the epigenetic regulation of CTCs.

Proteomic analysis of CTCs

While sequencing technologies have provided substantial advancements in the study of mutational frequencies, expression profiles, and now epigenetic features of CTCs, few technologies are available to measure CTC protein expression aside from antibody-staining approaches. Single-cell mass spectrometry is currently not technically feasible, but other technologies, including mass cytometry, permit the measurement of up to 40 different targets, including phosphorylation states in single cells (Spitzer and Nolan 2016). The technology uses antibodies coupled to heavy metal isotopes that allow precise and parallel quantitation of protein levels at the single-cell level. This technology allows for the careful measurement of signaling pathways within CTCs. However, the technology is limited by the availability of quality antibodies and the need to destroy the samples for analysis. This technology is currently a research tool that can provide insight into the biology of CTCs, and it remains to be seen whether it provides clinically useful information. While the development of single modalities to interrogate CTCs is expanding, the next step is to combine the modalities and analyze a single CTC for multiple parameters. Early work in this area has begun to combine metabolic assays with limited protein analysis and DNA sequencing of single cells (Zhang et al. 2015). Multimodal analysis promises to continue to expand and allow for the precise correlation of genetic, transcriptomic, proteomic, and metabolomic data within a single CTC.

Determinants of metastatic spread in CTCs

CTC access to the bloodstream

Cancer cells derived from a primary tumor can access the bloodstream in multiple ways, including direct intravasation into tumor-associated blood vessels or indirectly via the lymphatic system (Fig. 1). With either route, there can be both active and passive entry into the target vessels. For most cancers, including breast, colon, lung, and melanoma, the American Joint Committee on Cancer (AJCC) tumor, node, and metastasis (TNM) staging system uses the assessment of lymph node spread as a marker for a more advanced stage. This focus on lymph node status implies that lymph node positivity is a step in the progression of metastatic spread. However, there is little experimental evidence to suggest that metastatic cells necessarily have traversed the lymphatic system prior to forming a distant metastasis. It is unclear how much lymphatic spread contributes to distant hematogenous spread

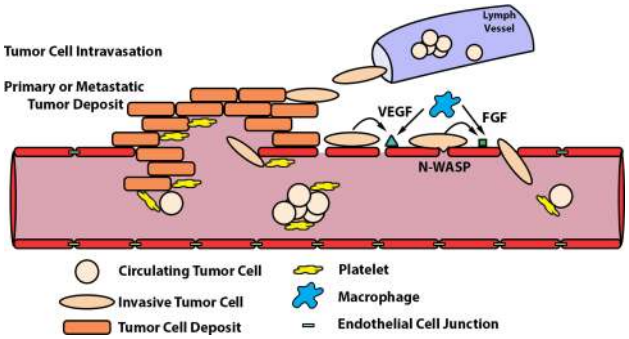

Figure 1. Tumor cell intravasation and the generation of CTCs: Tumor cells from primary and metastatic tumors intravasate either as single cells directly into blood or potentially as tumor cell clusters into the lymphatics, lymph nodes, and blood. The tumor cells can be shed passively into the circulation after a blood vessel is compromised or can actively intravasate without loss of blood vessel integrity. Macrophages in the tissue aid in the entry of CTCs into the bloodstream. VEGF, FGF, and other cytokines produced by macrophages and tumor cells lead to the loss of vascular junctions and increase permeability of the blood vessels. Invadopodia formation dependent on N-WASP in the tumor cell facilitates invasion through the endothelium.

or whether it is simply a marker of more invasive disease. In colon cancer, using analysis of somatic mutations in hypervariable regions to construct phylogenetic trees of lymphatic and distant metastases, 65% of distant metastases arose from a clone independent of corresponding lymphatic metastases (Naxerova et al. 2017). These observations suggest that the majority of distant metastases arises from a lineage distinct from lymphatic metastases. However, a recent study suggests that lymphatic remodeling induced by the primary tumor through secretion of MDK induces both lymphatic metastases and visceral metastasis, possibly through increased extravasation (Olmeda et al. 2017). Additional research is needed to define the factors that regulate lymphatic versus direct hematologic invasion of cancer cells. One determinant of metastatic spread to the lymph nodes is whether the cells are spreading as single cells or as a collective of grouped epithelial cells. Using intravital imaging to observe the movement of invasive cells in a model of breast cancer revealed that increased TGF-β signaling within the tumor cells favored single-cell motility, while collective migration continued despite inhibition of the TGF-β signaling (Giampieri et al. 2009). Remarkably, cells undergoing collective migration preferentially formed lymphatic metastases, while single invasive cells preferentially formed lung metastasis, presumably through intravasation directly into the blood.

Intravasation of tumor cells into proximal blood vessels generating CTCs has been difficult to model in vitro and assess in vivo. Presumably, direct access to the bloodstream can occur through compromised tumor-associated blood vessels and hemorrhage into the tumor. This passive shedding into the bloodstream is not well studied, and it is unclear how frequently it occurs. Active intravasation involves invading cells from the primary tumor invading the surrounding stroma directed by nutrient and growth factor gradients to blood vessels and then penetrating the wall of the vessel. Tumor cell-intrinsic factors are important regulators of intravasation and specifically the formation invadopodia regulated by the N-WASP protein. Inhibition of N-WASP through either a dominant-negative or shRNA down-regulation decreased the number of CTCs in a mouse and rat model of breast cancer (Gligorijevic et al. 2012).

In addition to cell-intrinsic features, the microenvironment and vasculature of the tumor can also contribute to CTC generation. Tumor-associated blood vessels display increased permeability and fragility that contribute to tumor cell access to the bloodstream (Fig. 1). The vascular dysfunction is due in part to the dysregulation of angiogenic signaling in the tumor, including FGF and VEGF (Huang et al. 2015), and inflammatory signaling, including endothelin B (Buckanovich et al. 2008) and PDL1 (Motz et al. 2014). The role of the vasculature in regulating access to the bloodstream and the generation of CTCs is exemplified by the study of decreased PHD2 expression in the vasculature, an oxygen-sensing molecule that targets the HIF transcription factor for degradation (Mazzone et al. 2009). *PHD2*$^{+/-}$ heterozygous deficient mice injected with *PHD2*$^{+/+}$ tumor cells form tumors with similar growth characteristics but show a dramatic decrease in intravasation and metastases. However, when the cells are directly injected in the bloodstream, they readily form metastatic lesions. This suggests that the vasculature can regulate tumor cell access to the bloodstream and the formation of CTCs.

In addition to the vasculature, the microenvironment of the invading tumor cell, including macrophages, also regulates CTC generation and intravasation. These complex, dynamic interactions are temporally and spatially localized. Specifically, tumor-associated macrophages have been identified as key regulators of tumor cell spread (Lin et al. 2006). TIE2-expressing macrophages promote tumor angiogenesis and metastasis and are often found in perivascular locations. Recent work has shown that VEGFA produced by these macrophages leads to transient vascular permeability, loss of vascular junctions, and increased intravasation locally at sites where tumor cells, macrophages, and blood vessels are in close proximity (Harney et al. 2015). Therefore, intravasation and the generation of CTCs are highly dynamic processed regulated by the tumor cells, the vasculature, and surrounding microenvironment.

Single CTCs versus clusters

CTCs are isolated from the blood as single cells or as clusters of two to 50 cells (Fig. 2). Multiple microfluidic devices have been developed to isolate the clusters without disrupting their integrity (Sarioglu et al. 2015; Au et al. 2017). Recent work has begun to investigate the features and functional role of CTCs within the clusters (Cheung and Ewald 2016). In a breast cancer mouse model, clusters are rare and represent <3% of the total CTCs. In a cohort of breast cancer patients with metastatic disease, 35% had

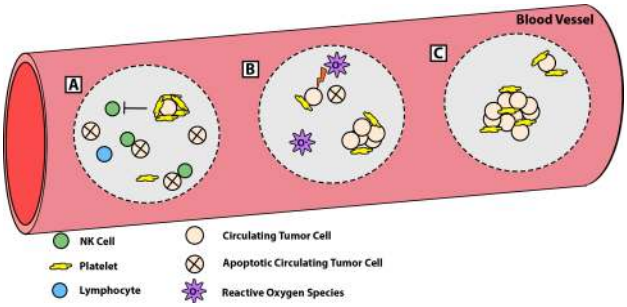

Figure 2. Feature of CTCs in the circulation: CTCs that have accessed the circulation are coated with platelets, which may protect them from the deleterious effects of the immune cells, including natural killer cells and lymphocytes (*A*); are subjected to the cytotoxic effects of reactive oxygen species (*B*); and/or travel as CTC clusters with increased metastatic propensity (*C*). These factors, together with other unknown mechanisms, affect the survival of CTCs in the blood.

detectable CTC clusters, while, in prostate cancer, 12.5% had detectable clusters (Aceto et al. 2014). CTC clusters have also been detected in non-small-cell lung cancer (NSCLC) (Hosokawa et al. 2013), colorectal cancer (Molnar et al. 2001), and melanoma (Luo et al. 2014). In breast cancer, the CTC clusters appear to be derived from oligoclonal clumps of primary tumor cells (Aceto et al. 2014) rather than the coalescence of single CTCs in the circulation, although the mechanism by which these clumps access the circulation is unclear. The half-life of the CTC clusters is likely on the order of minutes (estimated to be 6–10 min) and appears to be significantly shorter than for single-cell CTCs (25–30 min) (Aceto et al. 2014).

Independent of their generation, recent work has determined that the metastatic potential of CTC clusters is increased in a mouse model (Aceto et al. 2014). The mechanisms of this enhanced metastatic potential appear to be mediated in part through increased resistance to apoptosis. It is also possible that the reduced half-life of the CTC clusters in the circulation also aids in their survival and outgrowth. In a lineage tracing experiment in a spontaneous lung metastatic mouse model, polyclonal cell clusters were tracked from initial escape from the primary tumor to intravasation into the bloodstream to isolation of CTCs to micrometastases and macrometastases. At each stage, the clusters were found to maintain their polyclonal composition and have an increased metastatic potential dependent on keratin 14 expression in a subset of cells within the cluster (Cheung et al. 2016). In sum, CTC clusters are present in the bloodstream and contribute to CTC survival and likely metastasis.

Epithelial plasticity of CTCs

In the early steps of metastasis, epithelial cancer cells acquire the ability to separate from the primary tumor. This departure may occur as single cells or as clusters of cells (Friedl and Gilmour 2009) and requires the loss or alteration of cell-to-cell and cell-to-matrix interactions. These early steps of metastasis have been likened to a process described in development and wound healing, termed EMT (Nieto et al. 2016). In cancer, EMT-inducing signals have been implicated in the spread of cancer cells, although the precise role of EMT in metastasis is still under debate (Fischer et al. 2015; Zheng et al. 2015; Aiello et al. 2017; Ye et al. 2017). Oncogenic EMT has been associated with the acquisition of properties beyond invasion and migration and is implicated in tumor-initiating ability (Mani et al. 2008), resistance to drug treatments (Arumugam et al. 2009), immune evasion (Lou et al. 2016), and genomic instability (Comaills et al. 2016). Studies of EMT regulators are correlated with poor prognosis and advanced disease (Wu et al. 2015). Features of EMT are present in CTCs derived from carcinomas and contribute to multiple features of CTC biology (Yu et al. 2013; Micalizzi et al. 2017). Studies of CTCs have identified significant heterogeneity of epithelial and mesenchymal marker expression and the presence of biphenotypic cells that express markers of both cell lineages (Jolly et al. 2015). For instance, in metastatic breast cancer patients, CTCs analyzed with multiplexed RNA-ISH revealed a spectrum of epithelial and mesenchymal marker expression, demonstrating that EMT is a continuum. (Yu et al. 2013). Increased mesenchymal marker expression correlated with triple-negative and Her2-positive breast cancer and also was suggestive of therapeutic resistance. CTC heterogeneity for epithelial and mesenchymal markers has also been reported in pancreatic cancer (Ting et al. 2014) and prostate cancer mouse models (Ruscetti et al. 2015). Markers of EMT in CTCs have also been correlated with advanced disease or clinical outcomes in breast, colon, liver, and lung cancer (Yu et al. 2013; Wu et al. 2015). Epithelial plasticity is a critical feature of CTC biology, and future work will continue to define its role in metastasis.

Heterogeneity of CTCs

A key to understanding the biology of CTCs involves the study of CTC heterogeneity at the genetic, transcriptomic, proteomic, and metabolomic level. CTCs represent a dynamic cell population that is continually repopulated with cells from multiple sources that change significantly over the course of the disease and with treatment. Each blood sample containing multiple CTCs offers a snapshot of the global invasive cancer burden and reveals intrapatient and interpatient heterogeneity. Recent work using multiple tumor biopsies of the same tumor and sequencing of different regions of a resection specimen has demonstrated significant intratumoral heterogeneity for gene mutations, gene expression signatures, and overall cell ploidy (Gerlinger et al. 2012). With improvements in single-cell technologies, the clonal subpopulations of CTCs can be monitored, and we now have greater insight into the heterogeneity of CTCs.

With regard to genomic heterogeneity, mutations in *PIK3CA* in breast cancer CTCs have been evaluated as well as loss of heterozygosity detected among single CTCs and the presence of unique *PIK3CA* mutations in different CTCs from the same patient (Pestrin et al.

2015). Similarly, in lung cancer, *T790M* mutations were found in CTC samples from patients with primary tumor samples negative for *T790M* (Sundaresan et al. 2016). Multiple studies are now focusing on using CTCs and other blood-based diagnostics to track and monitor the development and evolution of mutationally distinct subclonal populations.

In addition to genetic heterogeneity of CTCs, heterogeneity of gene expression has also been studied. For example, in a heavily pretreated cohort, CTCs derived from estrogen receptor-positive metastatic breast cancer patients exhibit two distinct subpopulations based on expression of Her2 despite being negative for Her2 amplification (Jordan et al. 2016). The use of cell lines derived from breast cancer CTCs that maintain the two populations revealed phenotypic differences, including the proliferative rate and proteomic profile. The Her2- negative population exhibited increased Notch signaling, decreased sensitivity to chemotherapy, and increased sensitivity to γ secretase inhibitors. Interestingly, the two cell populations could interconvert within several cell doublings. Combination of chemotherapy and Notch inhibition potently targeted both populations, suggesting that the study of CTC heterogeneity can inform preclinical design of rational drug combinations. Additional studies using single-cell RNA-seq in prostate cancer and a mouse model of pancreatic cancer have also defined subgroups of CTCs (Ting et al. 2014; Miyamoto et al. 2015).

Metastasis-initiating cells have been proposed to exist as a subpopulation of the total CTC population, although markers of such a population have not been adequately defined (Celia-Terrassa and Kang 2016). In breast cancer, CTCs expressing $CD44^+CD47^+Met^{+/-}$ have been postulated to be enriched for a metastasis-initiating population and correlate with metastasis and survival (Baccelli et al. 2013). However, limiting dilutions have not been performed to determine the frequency of these rare cells in a functional assay. In work specifically focused on brain metastases, EpCAM-negative CTCs from metastatic breast cancer patients were isolated, cultured, and selected for a panel of markers representing a brain metastasis signature consisting of $Her2^+/EGFR^+/HPSE^+/Notch1^+$ (Zhang et al. 2013). This subpopulation of CTCs demonstrated a propensity to metastasize to the brain compared with unselected cell lines, suggesting that subpopulations of CTCs may determine organ tropism. The presence of a minor subpopulation of metastasis-initiating cells suggests that the majority of non-metastasis-initiating cells either dies in the circulation or distant tissue or remains in a dormant state.

CTC response to reactive oxygen species (ROS)

Upon entry into the bloodstream, CTCs are exposed to significant physical and biochemical stress that limits the survival of the vast majority of CTCs. In particular, increased oxygen tension in the circulation, loss of adherence to a matrix, and likely other factors contribute to increased ROS in CTCs (Fig. 2). The importance of cell adaptation to this oxidative stress is exemplified by recent work in melanoma, which showed that melanoma cells experienced significantly more oxidative stress in the blood and distant organs than in the subcutaneous tissue. It also demonstrated that metabolic changes in metastasizing melanoma cells increased their ability to tolerate oxidative stress (Piskounova et al. 2015). Knockdown of either ALDH1L2 or MTHFD1, important enzymes in the folate pathway, increased oxidative stress in the melanoma cells and inhibited distant metastasis. A second pathway via up-regulation of the β-hemoglobin (HBB) gene also is a mechanism by which CTCs tolerate oxidative stress (Zheng et al. 2017). HBB expression was observed in single-cell RNA-seq analysis of CTCs from patients with breast, prostate, and lung cancers. Analysis of cell lines revealed that increased ROS increases HBB expression and protected cells from ROS-induced apoptosis while decreasing the intracellular levels of ROS. Importantly, HBB expression and the antioxidant N-acetylcysteine increase metastatic potential of a breast CTC cell line. Together, these observations suggest that oxidative stress is an important obstacle for the survival and metastasis of CTCs, and CTCs use multiple pathways to adapt to the increased ROS. Future work will continue to define the role of ROS in the survival and metastatic ability of CTCs and also investigate the mechanisms of increased ROS production and changes in CTC metabolism.

CTC interaction with platelets

CTCs within the bloodstream are exposed to the components of the blood, and it has been recognized that these interactions affect CTC survival, gene expression, extravasation, and, ultimately, metastasis. As an example of this interaction, thrombocytopenic mice are protected from metastatic spread (Gasic et al. 1968). Platelet interactions with tumor cells through either direct interaction, secretion of platelet microvesicles, or release of platelet granules are implicated in resistance to apoptosis (Velez et al. 2014). The increased metastatic potential mediated by platelets has been hypothesized to be due to the adherence of platelets to the surface of the CTCs that prevent their recognition by the immune system (Fig. 2; Nieswandt et al. 1999) and potentially decrease the shear stress experienced by the tumor cells in circulation (Franco et al. 2015). A potential mechanism of platelet-induced enhancement of metastasis is based on the secretion of TGF-β from the platelets and direct cell surface interactions with CTCs. Coculture of colon and breast cancer cell lines with platelets activates the TGF-β pathway in the CTCs and promotes the up-regulation of mesenchymal markers and the down-regulation of epithelial markers, consistent with induction of an EMT. Cre-mediated deletion of TGF-β, specifically in the megakaryocytes and platelets, significantly reduced the metastatic potential of the colon cancer cells (Labelle et al. 2011). In addition to direct effects on the CTCs, platelets may also serve as a conduit through which CTCs can initially adhere, roll, and then arrest on the wall of a blood vessel. This interaction is mediated in part through selectins found on the surface of the platelets (Laubli and Borsig 2010) and

can be inhibited by an anti-coagulant (Mousa and Petersen 2009). Platelets have also been implicated in organ-specific metastases, particularly in the development of bone metastases, where release of lysophosphatidic acid from platelets stimulates the proliferation of tumor cells and the production of IL-6 and IL-8, activating osteoclast activity in the metastatic site (Boucharaba et al. 2004). More recently, RNA-seq analysis of single-cell CTCs has revealed the presence of gene signatures characteristic of platelets, including expression of CD41 and CD61, within a subset of CTCs (Ting et al. 2014). It is possible that these detected transcripts originate from platelets adhering to the surface of the CTC. Future work will continue to define the role of platelets in CTC signaling and metastasis.

CTC interactions with immune cells

The cells of the immune system have been recognized to both inhibit and promote tumorigenesis, depending on the cell type being analyzed and the context (Mohme et al. 2017). While the immunosuppressive microenvironment of the primary tumor has been well characterized (Rabinovich et al. 2007), CTCs do not benefit from the immunoprivileged features of the primary tumor. Instead, they are directly exposed to the diversity of immune cells in the blood. Therefore, it is not surprising that CTCs interact with the immune system and that these interactions affect immune function and CTC biology.

Innate tumor surveillance is a critical tumor suppressor and consists of coordinated activity of natural killer (NK) cells and macrophages. There is a correlation between the cytolytic activity of NK cells and the number of CTCs present in the blood of breast, colorectal, and prostate cancer patients (Santos et al. 2014). It is unclear whether decreased NK cell activity allows for a higher frequency of CTCs or whether increased CTCs modulate NK activity. Multiple mechanisms of CTC-induced NK cell inhibition have been proposed, including direct interaction of CTCs with killer cell immunoglobulin receptors (KIRs) on the NK cell surface, production of inhibitory cytokines, and increased platelet activation (Nieswandt et al. 1999; Mohme et al. 2017). Consistent with an immunosuppressive effect on NK cells in cancer patients, adoptive transfer of autologous NK cells after chemotherapy did not induce clinical responses in a small clinical trial despite the persistence of increased NK cells after transfer (Parkhurst et al. 2011). Therapeutic approaches to activate NK cell activity have been investigated, and a small molecule inhibitor of the TAM (Tyro3, Axl and Mer) kinases enhances NK-mediated killing of breast and melanoma cells and decreases metastasis in mouse models of aggressive cancer (Paolino et al. 2014). Macrophages also contribute to the innate immunosurveillance through the expression of Toll-like receptors (TLR), which can activate NK cell cytolytic killing (Bellora et al. 2014). Down-regulation of TLR2 and TLR4 in peripheral blood mononuclear cells correlates with increased numbers of CTCs in breast, colorectal, and prostate cancer patients (Santos et al. 2014). Therefore, down-regulation of TLRs on macrophages and NK cells is another potential mechanism of impaired tumor surveillance in patients with CTCs. In contrast to their immunosurveillance role, macrophages also play a critical role in establishing a premetastatic niche, particularly in the lungs. In a study using an intravital two-photon lung-imaging system, CTCs lodged in the capillaries of the lung begin to shed large microparticles, likely due to high shear forces (Headley et al. 2016). Shortly after CTC arrival in the capillaries, myeloid cells followed, including neutrophils, monocytes, macrophages, and dendritic cells, ingesting these microparticles. Macrophages that ingest these microparticles exhibit an activated phenotype and correlate with increased metastatic formation. Interestingly, the dendritic cells attracted to the arrested CTCs displayed an antimetastatic effect. Together, these observations demonstrate the complex interactions between immune cells and CTCs and the role in development of metastatic lesions.

In addition to the innate immune system, the adaptive immune system also plays an important role in tumor surveillance; however, the role of lymphocytes in immunosurveillance of CTCs is less clear. In metastatic breast cancer, there is a negative correlation between CTC count and lymphocyte count (De Giorgi et al. 2012), suggesting that the CTCs either modulate the presence of lymphocytes in the blood or increase as a result of low lymphocytes. Key to the function of CD8 T cells is the recognition of antigens bound to MHC class I molecules. If the T cell recognizes the presented antigen as foreign, it can activate its cytotoxic activity. To prevent their recognition by cytotoxic T cells, CTCs down-regulate the MHC class I receptor (Aptsiauri et al. 2007). Lymphocyte trafficking is also a critical regulator of metastasis, and a recent screen of 810 mutant mouse lines looking for host regulators of metastatic colonization identified deletion of Spns2 and lymphocyte trafficking to the lungs as important regulators of decreased metastatic burden (van der Weyden et al. 2017). Further work is needed to further define the role of the adaptive immune system in the immunosurveillance of CTCs.

Extravasation of CTCs and their colonization of distant organs

Although CTCs are a valuable source of information about the aggressiveness and metastatic potential of a cancer, there are additional barriers that must be overcome before an individual CTC or a CTC cluster gains the ability to form a metastatic lesion. Similar to earlier steps of the metastatic cascade, there is significant attrition of these cells at each step. Once a single-cell CTC or cluster has accessed the bloodstream and survived the initial shock of anchorage independence, shear stress, increased ROS, and exposure to platelets and immune cells, these cells must exit the bloodstream in an environment conducive to their continued survival and ultimately grow in a foreign microenvironment. These later stages of metastasis occur over vastly different time scales. The half-life of a CTC in the circulation has been estimated to be on the order of 25–30 min in a mouse xenograft

model (Aceto et al. 2014) and shorter for clusters. Metastatic lesions develop over a period of months to years, and, in some cancers, like breast cancer and prostate cancer, the disseminated tumor cells can survive for years before forming a macrometastatic lesion. Recent reviews (Dittmer 2017) have explored the regulation, survival, and activation of disseminated tumor cells, but it is likely that in the case of late relapses, the tumor cells must acquire additional genetic and epigenetic changes or significant alteration in the microenvironment to grow. In contrast, the exit from the bloodstream and initial colonization of a distant tissue rely on features of the CTCs.

CTC extravasation is thought to occur in a manner similar to extravasation of WBCs (Reymond et al. 2013). CTC extravasation occurs primarily in the small capillaries and at branch points between blood vessels based on in vivo imaging (Kienast et al. 2010). First, a single CTC or cluster forms an initial interaction with the blood vessel wall that is likely mediated through E-selectins expressed on the endothelium (Miles et al. 2008) and potentially facilitated by platelets (Fig. 3; Laubli and Borsig 2010). E-selectin is not typically expressed on endothelial cells but can be induced by cytokines released by the primary cancer (Hiratsuka et al. 2011). The initial interaction is low affinity and transient and likely facilitates a rolling motion of the CTC against the wall of the vasculature, similar to WBC adhesion. The cancer cell then arrests bound to the endothelium in a more stable interaction mediated through integrins, CD44, and MUC1, among other proteins. (Reymond et al. 2013). The binding of CTCs to the vascular wall is directed by not only the up-regulation of E-selectin on the endothelial surface but also numerous cytokines that originate from the target tissue and direct organ tropism. For example, CXCL12 is secreted by the stroma and can increase breast cancer cell adhesion to the vasculature and extravasation (Teicher and Fricker 2010). Once bound to the endothelium, CTCs penetrate the wall of the blood vessel by paracellular migration after breakdown of endothelial junctions. The opening of the endothelial junctions can occur in response to multiple factors, including TGF-β or VEGF produced by the tumor cell or accompanying immune cells (Hoeben et al. 2004; Drabsch and ten Dijke 2011). After opening the endothelial junctions, the tumor cells traverse the basement membrane and enter the stroma.

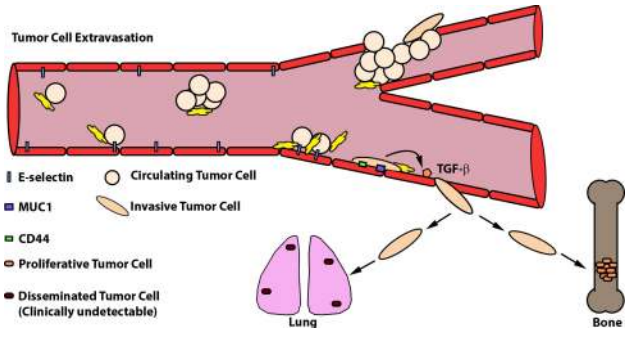

Figure 3. Extravasation of CTCs: CTCs that survive in the circulation can actively extravasate through the walls of the blood vessels or become lodged in branch points and capillaries. Lodged tumor cells can extravasate through compromised vessel walls. Other CTCs can form an initial interaction with E-selectin, expressed on the endothelial cells, that arrests the CTCs and allows them to form more stable interactions with MUC1, CD44, and integrins. Production of TGF-β by the tumor cells or platelets can facilitate opening of the endothelial tight junctions, allowing the CTCs to transverse the vessel wall. Extravasated CTCs can colonize and remain dormant or proliferate, giving rise to metastatic tumors.

Upon exit from the bloodstream, the CTCs must colonize and survive in the foreign microenvironment. The early stages of survival after extravasation are driven in part by genetic programs present in CTCs. For example, a subpopulation of CTCs has been identified in breast cancer that is characterized by expression of Her2, EGFR, HSPE, and Notch1 (Zhang et al. 2013). This population does not express EpCAM and therefore is not captured by the EpCAM-based CTC enrichment methods. Interestingly, the cells with this signature appear to selectively metastasize to the brain. In a second study, COX2 and the EGFR ligand HBEGF were identified as important regulators of the development of brain metastases in an in vivo metastasis model selected for preferential brain metastatic activity (Bos et al. 2009). These two genes were implicated in the extravasation of tumor cells through the blood vessels of the brain. A third protein, ST6GALNAC5, was shown to be necessary for transit across the blood–brain barrier. Additional gene signatures that correlate with lung and bone metastasis have also been reported (Kang et al. 2003; Minn et al. 2005), although these studies did not analyze CTCs directly. Together, these observations suggest that gene expression programs in the CTCs can direct the development of organ-specific metastatic spread and therefore are potentially amenable to evaluation in CTCs.

Clinical application of CTC characterization

With the substantial increase in the treatment options available to oncologists, it has become clear that concomitant with the rational design of new drugs comes the rational deployment of these treatments. Not every patient will respond, and, with few exceptions, most patients will develop resistance to these novel therapies and progressive disease. Biomarker-directed therapy and predictive testing of drug responses are key to efficient and effective treatment of individual cancer patients. While much of the information acquired to direct treatment is currently derived from primary tumor biopsies/resections or biopsies of a single metastatic lesion, it is clear that this approach of intermittent and often limited sampling of cancer is inadequate. The intrapatient cancer cell heterogeneity and rapid evolution of cancer, particularly under selective pressure, necessitate frequent and global evaluation of a patient's cancer. Recent advances in technology have allowed for the development of blood-based diagnostics that can assess an ever-increasing number of cancer-specific characteristics. The promise of these technologies has the potential to revolutionize cancer

detection, diagnosis, monitoring, and treatment with a noninvasive test.

For early detection, CTC evaluation in high-risk patients has the potential to identify neoplastic disease earlier than standard methods of imaging or blood-based biomarkers. For example, in patients with COPD, circulating epithelial cells were detected 1–4 yr prior to detection of lung nodules on screening CT scans (Ilie et al. 2014). Importantly, no circulating epithelial cells were detected in a small cohort of smokers and healthy donors without COPD or cancer. In a second study, isolation of CTCs followed by an RNA-based digital PCR analysis for liver-specific transcripts provided orthogonal information with the standard biomarker AFP in a population of patients with HCC (Kalinich et al. 2017). Together, these results suggest that CTC analysis may be a sensitive screening method.

In patients with confirmed neoplastic disease, CTCs can provide prognostic information and mutational information that can direct treatment and provide predictive drug responses. For instance, in prostate cancer, conversion from the unfavorable risk group to the favorable group based solely on CTC enumeration had improved survival (6.8 mo to 21.3 mo) (de Bono et al. 2008). In addition, AR splice variant 7 (AR-V7) analysis of CTCs in patients treated with the AR antagonists abiraterone and enzalutamide revealed that patients with this splice variant displayed lower response rates and decreased progression-free survival and overall survival (Antonarakis et al. 2014). Prospective studies are needed to confirm these results and validate AR-V7 in CTCs as a predictive biomarker. The clinical applications of CTC characterization are also evident from studies in lung cancer where EGFR mutations can be detected, specifically the T790M mutation, which correlates with resistance to first- and second-generation EGFR inhibitors (Maheswaran et al. 2008) and has the potential to direct therapy to a third-generation inhibitor. Together with ctDNA-based genetic monitoring, the ability to analyze cell-based components will greatly enrich the tools available to guide therapeutic choices. Ongoing prospective studies are needed to validate and provide clinical evidence for the value and benefits of CTC-based diagnostics as well as other blood-based markers.

Future directions

Advances in cancer treatment will continue to expand with new targeted and immunotherapies on the horizon and an emphasis placed on precision and personalized medicine. In parallel with the development of these exciting new therapies, advances in companion diagnostics and biomarkers will be critical to the rational use of these treatments and their success. Currently established biomarkers are derived primarily from biopsy or resection specimens, which do not allow for repeated sampling, harbor some risk to the patient, and can be prone to sampling errors. CTCs and other blood-based diagnostics offer an opportunity to gain important molecular and cellular information about a cancer over time, providing "real-time" prognostic and predictive information. Numerous clinical trials are open or in development using CTCs in cancers ranging from breast (NCT01048918) and melanoma (NCT02828345) to prostate (NCT01961713) and colon (NCT03033927) cancer. Most of the currently open trials are using CTC enumeration as the primary measure, but we expect that future trials will interrogate CTC mutations, gene expression, and epigenetic properties as potentially more informative clinical parameters. The clinical utility of CTCs has the potential to allow for more frequent and less invasive monitoring of disease burden, with the possibility of directing treatment decisions. CTCs also have the distinct advantage of allowing for functional and cellular-based studies, which have already provided valuable information about the process of metastasis, including the generation of CTCs, their survival in the bloodstream, their interaction with blood components, and their exit from the blood to generate distant lesions. These studies will continue to increase our knowledge of the metastatic process, with the hope of identifying new vulnerabilities that can target the lethality of cancer metastasis.

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