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Orchestration of Collective Migration and Metastasis by Tumor Cell Clusters

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Abstract

Metastatic dissemination has lethal consequences for cancer patients. Accruing evidence supports the hypothesis that tumor cells can migrate and metastasize as clusters of cells while maintaining contacts with one another. Collective metastasis enables tumor cells to colonize secondary sites more efficiently, resist cell death, and evade the immune system. On the other hand, tumor cell clusters face unique challenges for dissemination particularly during systemic dissemination. Here, we review recent progress toward understanding how tumor cell clusters overcome these disadvantages as well as mechanisms they utilize to gain advantages throughout the metastatic process. We consider useful models for studying collective metastasis and reflect on how the study of collective metastasis suggests new opportunities for eradicating and preventing metastatic disease.

1. INTRODUCTION

Metastasis, the process by which cancer cells disseminate and colonize distant organs, is responsible for the majority of the estimated 9.9 million cancer-associated deaths occurring worldwide every year (1, 2). This complex process involves the orchestration of sequential steps that must all be completed successfully for metastasis to emerge. Tumor cells must detach from the primary tumor and disseminate to a secondary site, in many cases far from the initial site of cancer. Thereafter, disseminated tumor cells (DTCs) must rapidly adapt to a newfound, often hostile, environment. Perhaps most difficult of all, upon arrival, tumor cells must construct a more favorable metastatic niche to survive and ultimately proliferate. Partly due to heterogeneity in timeline, mechanism, and pattern of spread, metastasis remains a challenge to treat clinically and to study biologically.

Metastatic dissemination has often been thought to be primarily completed by cells traveling alone. This is premised on observations that tumor cells become more migratory when they lose cell–cell attachment, that tumor cells in circulation are mostly single cells, and that single DTCs can be found throughout a cancer patient's organs. However, there is evidence that tumor cells can metastasize cohesively as a clump, embolus, or cluster of tumor cells rather than as single cells. In the 1970s, studies independently demonstrated that tumor cell clumps have greater metastatic seeding potential compared with single tumor cells (3, 4). Meanwhile, observations in a host of developmental and cancerous states have firmly established that cells can capably migrate while maintaining cell contacts during morphogenesis and cancer invasion (5). It has become increasingly clear that tumor cell clusters are found in cancer patients' bloodstreams (and bone marrow) in multiple cancer types and that the presence of circulating tumor cell (CTC) clusters is associated with worse prognosis (6–11). These studies and others have provided clues to a mode of collective metastasis in which groups of tumor cells execute the metastatic process as clusters of cells.

In this review, we discuss the recent progress toward understanding the molecular mechanisms of collective metastasis and its consequences. We first highlight the weight of clinical and experimental evidence supporting collective metastatic seeding. We discuss the emerging evidence for diverse intercellular interactions within tumor cell clusters, endowing cancer cells with enhanced metastatic aggression. At the same time, clusters may also face unique disadvantages at early steps in metastasis, particularly while navigating systemic circulation. We discuss the diversity of mechanisms involved in tumor cell collective migration and discuss how dissemination may be accomplished by a tumor cell cluster. We highlight how different models have supported collective metastasis research and identify avenues for future technological development. While collective metastasis remains a field in its early stages, we propose that understanding how collectivity arises and how it changes tumor cell behavior during metastasis is essential in the quest to prevent and eradicate metastatic disease.

2. EVIDENCE FOR COLLECTIVE METASTATIC SEEDING

For the purposes of this review, we define collective metastatic seeding as the completion of dissemination, seeding, and outgrowth phases of metastasis by a cluster of at least two tumor cells. As discussed below, there is strong evidence that tumor cell clusters can be found in cancer patient blood and that their presence is associated with worse prognosis. These clusters, despite their low frequency compared with single tumor cells, make large contributions to productive metastases.

2.1. Circulating Tumor Cell Clusters Are Detected in Many Cancers, and Their Presence Is Associated with Poorer Patient Outcomes

The ability to isolate and characterize circulating tumor cells provides a window into the properties of disseminating tumor cells in transit. Because blood draws are relatively noninvasive and

Table 1 Percentage of patients with single CTCs and CTC clusters, from selected studies

Reference	Cancer type	% of patients with single CTCs	% of patients with CTC clusters	Number of patients
Jansson et al. 2016 (145)	Breast[b]	NR	27.0%	52
Wang et al. 2017 (8)	Breast[b]	60.2%	16.4%	128
Larsson et al. 2018 (10)	Breast[a]	52.0%	20.0%	156
Paoletti et al. 2019 (24)	Breast[a]	52.0%	19.0%	266
Costa et al. 2020 (146)	Breast[a]	57.4%	25.9%	54
Divella et al. 2014 (147)	Colorectal	67.0%	32.0%	103
Zheng et al. 2017 (148)	Gastric[a]	43.0%	26.0%	81
Hou et al. 2012 (6)	Lung (SCLC)[a]	85.0%	32.0%	97
Long et al. 2016 (7)	Melanoma[a]	51.0%	34.0%	128
Lee et al. 2017 (149)	Ovarian	98.1%	59.2%	54
Chang et al. 2016 (150)	PDAC[a]	81.0%	81.0%	19
Okegawa et al. 2018 (151)	Prostate[a]	NR	50.0%	98
Mu et al. 2015 (9)	Breast[b]	31.3%	17.4%	115
Carlsson et al. 2017 (11)	Prostate[a]	Blood: 33.0% Bone marrow: 23.0%	NR NR	141
Diamantopoulou et al. 2022 (23)	Breast	100.0%	30.0%	30

[a]Baseline analysis of CTC cluster presence.
[b]Longitudinal time-dependent analysis of CTC cluster presence.
Abbreviations: CTC, circulating tumor cell; NR, not reported; PDAC, pancreatic ductal adenocarcinoma; SCLC, small-cell lung cancer.

frequently obtained from cancer patients, CTCs are promising for monitoring therapy response and disease evolution, as reviewed extensively elsewhere (12). Although studies of CTC abundance vary by tumor type, tumor stage, blood collection site, and isolation strategy, a common conclusion is that the presence of CTCs correlates with worse progression-free survival (PFS) and overall survival (OS) in most common human cancers (13, 14).

Beyond individual CTCs, newer technologies and analytical methods have identified CTC clusters in many metastatic cancer types (15–19). These assays have defined CTC clusters as a group of two or more cohesive cells. Such CTC clusters are consistently rarer than single CTCs. Among studies investigating both single CTCs and CTC clusters, between 1% and 10% of CTC events are clusters (11). Between 43% and 98% of patients with metastatic disease have detectable single CTCs in their bloodstream, with detection defined most commonly as >5 CTCs per blood draw (see **Table 1**). In comparison, the same studies found one or more CTC clusters per blood draw in 16–59% of patients (see **Table 1**). While most of these studies have focused on patients with advanced-stage disease, there is also clinical evidence that CTC clusters can be detected in early-stage cancers, prior to or during definitive surgical resection (20–23).

Patients with CTC clusters detected in their bloodstream have significantly poorer PFS and OS compared with patients with only single CTCs (6–10). Hazard ratio (HR) is a common way to quantify the difference in risk between groups. For example, if the HR of death is 2.0, this means the rate of death is twice as high in one group than in the other. In available studies, the HR for OS for patients with CTC clusters compared with patients with only single CTCs ranged from 2.9 to 15.1 (6, 8–10). However, two limitations should be acknowledged when interpreting these studies. First, CTC clusters tend to occur in patients with the most CTCs, as reflected in one large reanalysis of prospectively collected CTCs using CellSearch in metastatic breast cancer patients (24). When enumeration takes into account a higher CTC threshold, the presence of

CTC clusters is no longer independently prognostic. This could indicate that CTC clusters may simply be an indicator of high CTC abundance. Whether underlying cellular biological processes driving CTC and CTC-cluster dissemination are shared remains to be tested. Second, the entry point of enrollment for many studies is not uniform. The frequency of CTC clusters varies with disease progression and is highest in patients with treatment-resistant and refractory cancers (8, 9, 24, 25). Whether CTC clusters are a cause or consequence of therapy resistance is incompletely understood, though one recent study has suggested that resistance to endocrine therapy shifts metastatic breast cancer cells toward a clustered phenotype (26). Despite these confounding factors, the presence of CTC clusters can be confidently interpreted as a poor prognostic factor.

At present, the true frequency of CTC clusters remains uncertain, and the optimal approach toward identifying CTC clusters is not yet understood (discussed in the sidebar titled Challenges in Circulating Tumor Cell Cluster Detection and Isolation). For now, we point out that relying on different qualifications and cell surface markers to define CTCs inherently means that the definitions of CTC clusters vary by study. In addition, it is difficult to tell if CTC clusters are being undersampled because they are breaking apart or becoming trapped during the CTC isolation

CHALLENGES IN CIRCULATING TUMOR CELL CLUSTER DETECTION AND ISOLATION

CTCs are rare, dynamic, short-lived entities that occur at frequencies of <1 CTC per 10 million leukocytes and 100 million red blood cells (28). This makes the enrichment, isolation, and detection of CTCs extremely challenging. At present, only one CTC detection method, CellSearch, is FDA-approved to detect CTCs in breast, prostate, and colorectal cancer patients to help determine prognosis (12). CellSearch is one of many surface marker–based methods that relies on the expression of EpCAM to detect CTCs. A known limitation of EpCAM-based detection is its inability to detect CTCs that do not express EpCAM. An extension of this problem germane to CTC clusters is that tumor cells show heterogeneous expression for different markers (7, 29). Further, CTC clusters can occur in complexes with immune cells such as neutrophils (30), which can interfere with CTC cluster isolation by affinity-based methods such as negative depletion of $CD45^+$ immune cells. One approach that may overcome these challenges is use of label-free methods that select for CTCs and clusters based on size and deformability. Microfluidic devices enable the recovery of heterogeneous CTC populations including clusters and do not rely on surface markers such as EpCAM (16, 27, 31, 32). The fact that most CTC clusters are doublets and therefore may have size characteristics not much different from single CTCs is a challenge in these methods. Further, microfluidic devices that enforce certain channel size dimensions or impose critical flow rates may induce shearing that disaggregates CTC clusters. Perhaps the simplest approach is red blood cell lysis followed by affixing residual cellular fraction to slides and staining these slides for markers such as cytokeratin and CD45 that can be analyzed by microscopy. This method, however, makes it more difficult to process CTCs for downstream analyses or cultivation (33). At present, it remains a fundamental challenge that CTC technologies are developed and optimized using simulated CTCs generated via spike-ins of human cancer cells into blood samples. These technologies are then tested in human patient populations where the true properties of CTCs are unknown and depend on tumor type, disease stage, and follow-up time. Such complications make it difficult to determine the most efficient, catchall, unbiased CTC detection and isolation methods. Mouse and other patient-derived xenograft animal models that spontaneously and reproducibly generate CTCs could be valuable for these efforts, but CTCs in these models are even more rare and short-lived due to their small circulating blood volume. Better models that allow for sampling and analysis of large numbers of CTCs collected in an unbiased manner could be pivotal to gain a deeper understanding of CTC and CTC-cluster characteristics and variations.

process. CTC clusters could also be rendered invisible to detection through filtration in a more proximal capillary bed. CTC abundance also varies dynamically and can vary on a circadian level (23). The ability of CTC clusters to remain intact during isolation is dependent on many factors including the shear stress experienced by CTC clusters during the isolation process. In recent years, various technologies have been designed specifically to maintain and recover clusters from the blood (16, 27). The importance of CTC-cluster size (how many cells are in a CTC cluster) also remains understudied. One label-free technology designed to isolate CTC clusters found that 30–40% of patients with metastatic breast cancer, prostate cancer, or melanoma had CTC clusters and that CTC-cluster size ranged from 2 to 19 cells. CTC-cluster cell count trended toward exponential distribution, with many more 2-cell clusters than larger clusters (16). A few studies have suggested that larger CTC-cluster sizes could provide advantages beyond 2-cell clusters (8). In one study of metastatic breast cancer patients, authors found that the OS HR is 14.5 for patients with clusters containing at least 3 cells compared with patients with no CTCs, whereas the OS HR is 7.96 for patients with 2-cell clusters. Whether larger CTC clusters correlate with worse prognosis will require larger well-powered studies using technologies designed to capture clusters.

2.2. The Outsized Potential of Tumor Cell Clusters for Metastatic Seeding

Early CTC-cluster studies in the 1970s reported that when tumor cells were separated into clusters or single tumor cells and injected intravenously into mice, tumor cell clusters produced between 3- and 25-fold greater lung metastases (3, 4). More recent studies have shown similar effects with between a 2- and 500-fold increase in metastasis formation using a variety of cancer models (breast, melanoma, and colon) and delivery routes (intravenous, intracardiac, intraperitoneal, and retro-orbital) (17, 34–40). (Model systems for collective metastasis are discussed further in the sidebar titled Models for Studying Collective Metastasis.)

These experimental metastasis models support the notion that tumor cell clusters have robust metastatic potential. However, because CTC clusters are rare in patients and in mouse models,

MODELS FOR STUDYING COLLECTIVE METASTASIS

A variety of 2D and 3D ex vivo systems, bioengineered microfluidic devices, and animal models have been useful to define the cellular and molecular processes governing collective migration of tumor cells. Whereas developmental migrations are highly reproducible processes in developmental space and time, tumor cell dissemination and metastasis are highly stochastic, generally low probability, spatiotemporal events that occur within complex heterogeneous microenvironments. For this reason, great care must be taken when extrapolating conclusions drawn in low-complexity but more tractable model systems to more complex and potentially realistic systems. At the same time, in vitro model systems can reveal what is possible, for example, demonstrating the ability of collectively migrating tumor cells to invade and remodel the local microenvironment. The design of more complex in vitro models that are assembled into a realistic model system—such as incorporating organoids, different matrix environments, metabolic constraints, and tumor microenvironment cellular components—is an area of ongoing investigation. Animal models including zebrafish, chick allantoic membrane, and mouse models are also frequently used to establish in vivo significance. Nonetheless, the route and delivery of tumor cells can significantly impact metastatic outcome, when intravenous, intracardiac, or orthotopic transplantations are performed. Perhaps the greatest complicating factor is the significant heterogeneity in metastatic behavior between different tumor models and cancer types. The development of better patient-derived cancer models [e.g., patient-derived xenografts (PDXs), PDX organoids, and CTC-derived tumor lines] holds promise to uncover universal and subtype-specific metastatic properties.

it is possible that individual CTCs nonetheless contribute to the majority of metastatic seeding. How, then, can we determine the contribution of multicellular seeding? One experimental approach that has proven useful is lineage tracing with multicolor fluorescent labeling of tumor cells in animal models of metastasis including mice, zebrafish, and fruit flies (17, 34, 35, 38, 41–48). In these experiments, multicolor primary tumors are generated by transplanting tumor cells into a tissue of origin or through genetic models of metastatic cancer. These primary tumors are composed of two or more tumor clones expressing different fluorescent reporters that are maintained through division. Metastases develop over time and are then examined by microscopy. If metastases arise exclusively from individual clones, then only single-color metastases would be expected. If metastases are multicolored, then at some point in their development they arose from two or more distinct clones. Using this approach, multiple groups have reported that the majority, in some cases more than 90%, of metastatic seeding events are polyclonal. The multicolor labeling approach can be further extended by increasing the number of colors or by using genomic barcoding (34, 42–44, 46). A limitation of these methods at present is the unequal proportion of different clones in primary tumors, a factor that tends to decrease overall clonal diversity and hence the power to detect multicolor metastases. Nonetheless, these studies suggest that only a small number of clones, most commonly two, contribute to polyclonal seeds.

Polyclonal metastatic seeding could arise from either multicellular seeding from a CTC cluster (which exited and seeded as a cluster) or via serial seeding of individual tumor cells over time (49, 50). To answer this question, different studies have expanded on multicolor tracing experiments by employing transplantations where different tumor clones are spatially separated such that aggregation can only occur after entry into circulation. For example, in the case of breast cancer mouse models, the left and right mammary glands are transplanted so that one color is on the left and one is on the right. In such cases, a number of studies have indicated that the frequency of serial seeding is small, ranging from 0 to 14% of events (17, 34, 38, 43). These numbers are much smaller than when primary tumors are a mixed population of clones, where up to 97% of metastases are polyclonal (44, 34). Given the outcomes of these studies, we can conclude that polyclonal metastases most likely arise primarily from seeding by a cluster originating from the primary tumor.

These studies have highlighted additional caveats when detecting polyclonal metastasis. One such is clonal sweeps, in which one tumor clone gains a fitness advantage such that it takes over the entire tumor (51). This is particularly apparent when fluorescent marker tracing begins early in models that encompass early and late tumor progression (35, 41). When fluorescent markers are induced early, tumors show a tendency to be dominated by a single clone, whereas induction at later points results in greater clonal mixing and multicolored metastases. Second, it is important to distinguish studies in which different fluorescent reporters are used to tag tumor cells from studies in which different fluorescent reporters mark tumor cells with distinct functional properties (45, 48). For example, Kok et al. (48) show through a cotransplantation experiment into the spleen that nonmetastatic colorectal cancer cells codisseminate to the liver with metastatic cells, inducing the generation of a supportive fibrotic niche. Likewise, more invasive (INV) melanoma cells cooperate with less invasive but more proliferative (PRO) melanoma cells to form polyclonal metastasis in zebrafish models (45). Third, different studies also suggest that the preference for polyclonal seeding and monoclonal seeding could depend on organ site (35, 44). It remains to be determined if the tumor microenvironment shapes whether sites are more receptive to single or collective seeding.

In conjunction with experimental observations, genomic studies in cancer patients have demonstrated evidence for frequent polyclonality at every step of metastatic progression (52–56). These clinical data support the model that polyclonal metastatic diversity observed in metastases

could potentially arise from seeding by tumor cell clusters composed of genetically distinct clones. It should be acknowledged that it is not knowable in these patients whether polyclonal seeding events arose from multicellular seeding of clusters or serial seeding of individual tumor cells. Additionally, the clonal diversity afforded by a small multicellular seed could be minor and therefore highly susceptible to a clonal sweep (51), which may reduce detection of polyclonal seeding events. Therefore, inference about the migration history from genomic history may not yield a single answer but may point to the potential for multiple compatible seeding histories (57). We expect that further advances in spatial and temporal sequencing will provide greater understanding of multicellular seeding and its contribution to polyclonal metastases in cancer patients.

3. MECHANISMS BY WHICH TUMOR CELL CLUSTERING PROMOTES METASTATIC SEEDING

3.1. Benefits of Clustering at Different Stages of Metastatic Seeding and Outgrowth

Tumor cells in circulation are bombarded by multiple insults that act as barriers to survival and metastasis formation (58, 59). These include mechanical insults such as fluid shear stress (FSS), which can shred tumor cells in high-flow vessels. During circulation, some tumor cell clusters experience loss of cell–cell and cell–matrix attachments necessary for tumor cell survival, leading to anoikis and metabolic rewiring (60, 61). Clusters in circulation are also exposed to oxidative stress, which can lead to ferroptosis and other death pathways (62, 63). Further, clusters are vulnerable to immune attack in circulation or immediately thereafter when lodged in the distant organ (43, 64, 65). Statistically speaking, completing all these steps is therefore highly unlikely. Emerging studies indicate that tumor cell clusters potently change these odds.

3.1.1. Resisting fluid shear stress. FSS, induced by liquid flow, is defined as the internal frictional force between moving layers in laminar flow (58). CTCs experience FSS in transit, of which the magnitude and duration of exposure can affect the chances of CTC survival. CTCs subjected to high FSS can induce mechanical stress, cell fragmentation, and death, while CTC clusters are more resilient to mechanical forces (66–68). This resilience occurs not only as a function of superior physical integrity through cell–cell adhesion but also because traveling as clusters increases drag force, decreases the speed of CTCs traveling as clusters, and promotes their intravascular arrest (58, 66).

3.1.2. Resisting cell–cell and cell–matrix detachment–induced cell death. Attachment to the extracellular matrix (ECM) is important for cell survival, and upon loss of anchorage to the ECM, cells undergo a form of programmed cell death called anoikis (59). The formation of tumor cell clusters rescues tumor cells from anoikis through maintenance of cell–cell attachment. Cell–cell attachment can protect cells from additional stressors encountered in circulation via signaling pathways. These include nectin-1/nectin-4 binding in adjacent tumor cells, which drives integrin β4/SHP-2/c-Src activation (63), E-cadherin-dependent reactive oxygen species protection (69), and DNA methylation to induce stemness transcription factors (70). These studies advocate the development of therapies that break apart tumor cell clusters to mitigate their metastatic advantages.

3.1.3. Avoiding immune attack. There is still much that is unknown about CTC avoidance of immune modulation, especially whether there are differences between single cells and clusters. However, there is evidence of CTC cluster–specific mechanisms to avoid immune surveillance

from natural killer (NK) cells. Infiltration of NK cells into tumors and high expression of NK cell receptor genes are correlated with better patient prognosis, indicating that NK cells can target tumor cells (71). Lo et al. (43) recently found that selectively depleting NK cells increased monoclonal but not polyclonal metastases, suggesting that NK cells can effectively kill single tumor cells but not clusters. Tumor cell clusters were revealed to have increased expression of cell–cell adhesion and epithelial genes and decreased expression of NK cell–activating ligand. These studies hint at the existence of cluster-specific immune evasion mechanisms, potentially conferring tumor cell clusters an advantage throughout the metastatic process. There is also increased interest in NK-cell therapy for cancer patients with advanced disease, in which NK cells' ability to kill CTCs and DTCs is exploited for therapy (72, 73). Gaining a deeper understanding of cluster-specific avoidance of NK cell killing could be key to developing such therapies to target tumor cells while in circulation.

3.1.4. Increased metastatic outgrowth. As stated above, there is a wealth of evidence that circulating clusters are more likely than single cells to seed metastatic lesions that grow out quickly (40). This concept is mirrored in some 3D culture models, where PyMT tumor cell clusters survive longer and proliferate up to five times more quickly than single cells, an effect that increases with the number of cells in the cluster (39). Several studies have revealed that clusters have several advantages over single cells that have arrived in the distant organ, including increased stemness and intercellular signaling that stimulates tumor outgrowth, and cooperative polyclonal interactions.

One such signaling mechanism has been described in which minor subclones of breast cancer cells expressing IL11 and FIGF signal to one another as well as to stromal and immune cells to promote metastasis (74). In another case, integrin–ECM adhesion mechanisms were utilized by subclones to increase proliferation. MCF10A PIK3CA mutant subclones upregulate fibronectin, promoting growth of Her2 mutant subclones. Inhibition of integrin–fibronectin binding reduces the proliferative benefits of growing the two clones together (75).

Recently, our lab has found that when breast cancer cells travel throughout the body together as clusters of cells, instead of as individual cells, they are >100 times more likely to form metastases. In this work, we found that one reason tumor cells metastasize better together is because breast cancer cells adhere to one another and form intercellular spaces between their cell–cell adhesions, which we termed nanolumina. We found that these nanolumina act as shared reservoirs for the growth factor epigen. Blocking tumor cell clusters from expressing and sharing epigen greatly reduced their growth in vitro and in vivo, suggesting that nanolumina and the cell–cell signaling they generate play a key role during metastatic outgrowth (39). In addition, a recent study showed that a single clone isolated from an ovarian tumor sample was the primary population in metastases. However, when injected alone, this clone was incapable of forming metastatic lesions. It was uncovered that this clone expressed high levels of ERBB2 and was only able to seed metastases in the presence of a clone expressing high levels of AREG or when exposed to exogenous AREG (76). Together these studies implicate a role for collective family signaling in tumor seeding and metastatic outgrowth.

Consistent with the model of cooperation between tumor cell clones, other studies also have found scenarios where less-fit subclones can metastasize with more-fit metastatic clusters to form distant polyclonal metastases. For example, in colorectal carcinoma cells, more aggressive metastatic cells traveled with and enabled the seeding of less-fit metastatic cells by priming the niche via fibrosis (48). Similarly, a melanoma cell line clone that is highly invasive and capable of metastasis can adhere to a more proliferative clone, delivering it to the metastatic site and increasing growth potential (45).

3.2. Composition of Tumor Cell Clusters Promoting Metastatic Seeding and Outgrowth

There has been significant interest in defining various cell adhesion complexes coupling tumor cells together, enabling cluster-driven metastasis. In addition, there is growing recognition that clusters are composed of phenotypically, and in some cases genotypically, distinct cell types (**Figure 1**). Understanding how these cells each contribute to collective metastases can uncover mechanisms behind metastatic survival and outgrowth.

3.2.1. Cell-cell adhesion. The types of cell adhesion complexes present in tumor cell clusters vary by tumor type. The most common cancers are epithelial in origin and accordingly contain adherens junctions, desmosomes, and tight junctions, all vital for maintaining cell–cell contact (5). In some cases, tumor types such as melanoma can be more reliant on adhesion complexes of other types such as neural cell adhesion molecules. Given the rarity of CTC clusters, a systematic understanding of the adhesion complexes most important for CTC-cluster integrity and metastasis is necessary.

Nonetheless, a raft of studies suggests several common themes important to highlight. The first is that multiple studies in epithelial tumors have implicated the metastasis-promoting role of various components of desmosomes, which strongly anchor cell–cell contacts. Loss of junction plakoglobin, a key intracellular component of the desmosome, reduces CTC clustering and metastatic potential (17). Likewise, expression of desmogleins, desmocollins, and plakophilins (such as DSG1, DSC2, and PKP1) has been shown to support clustering and metastasis formation (68, 77). It therefore stands to reason that desmosomal targeting could have profound effects on CTC-cluster integrity and likely on the integrity of micrometastases in distant organs. In addition, disruption of desmosome adhesion could alter the frequency and type of tumor dissemination. For example, in one breast cancer mouse model, loss of DSG2 enhanced single-cell CTC dissemination while its reexpression induced more CTC clusters (77).

The expression of various cell adhesion molecules not only supports clustering but also induces downstream signaling that can affect the survival and outgrowth of tumor cells. E-cadherin contacts are critical for adherens junction formation and play important structural and signaling roles in migrating tumor collectives. Accordingly, constitutive loss of E-cadherin is accompanied by marked reductions in metastasis formation (69, 78). Nectin-1 (PVRL-1) and nectin-4 (PVRL-4) binding between adjacent tumor cells is a potent inducer of anchorage-independent survival and growth, driving ECM-independent integrin β4/SHP-2/c-Src activation to prevent apoptosis (79). There is further evidence that α6β4 integrin signaling activation and subsequent GPX4 expression prevent lipid peroxidation and ferroptosis (63).

Cell adhesion confers an additional advantage to tumor cells by reducing the distance needed for productive intercellular communication. Tumor cells can communicate by passing signaling molecules to neighboring cells via gap junctions at cell–cell contacts. Connexins, a major component of gap junctions, have been implicated in many aspects of metastasis, although the specifics of intercellular signaling via gap junctions during collective metastasis need further investigation (80). In addition, the physical binding of tumor cells to one another can induce intercellular spaces that act as sites of collective intercellular signaling (39). As described above, breast tumor cell clusters secrete and concentrate growth factor into the spaces, such that, regardless of the microenvironment these clusters find themselves in, collective signaling can be maintained, encouraging survival and growth at the metastatic site (39). In some cases, the signaling molecules between cells are packaged into exosomes (81) or passed between cells via cytoplasmic transfer (82).

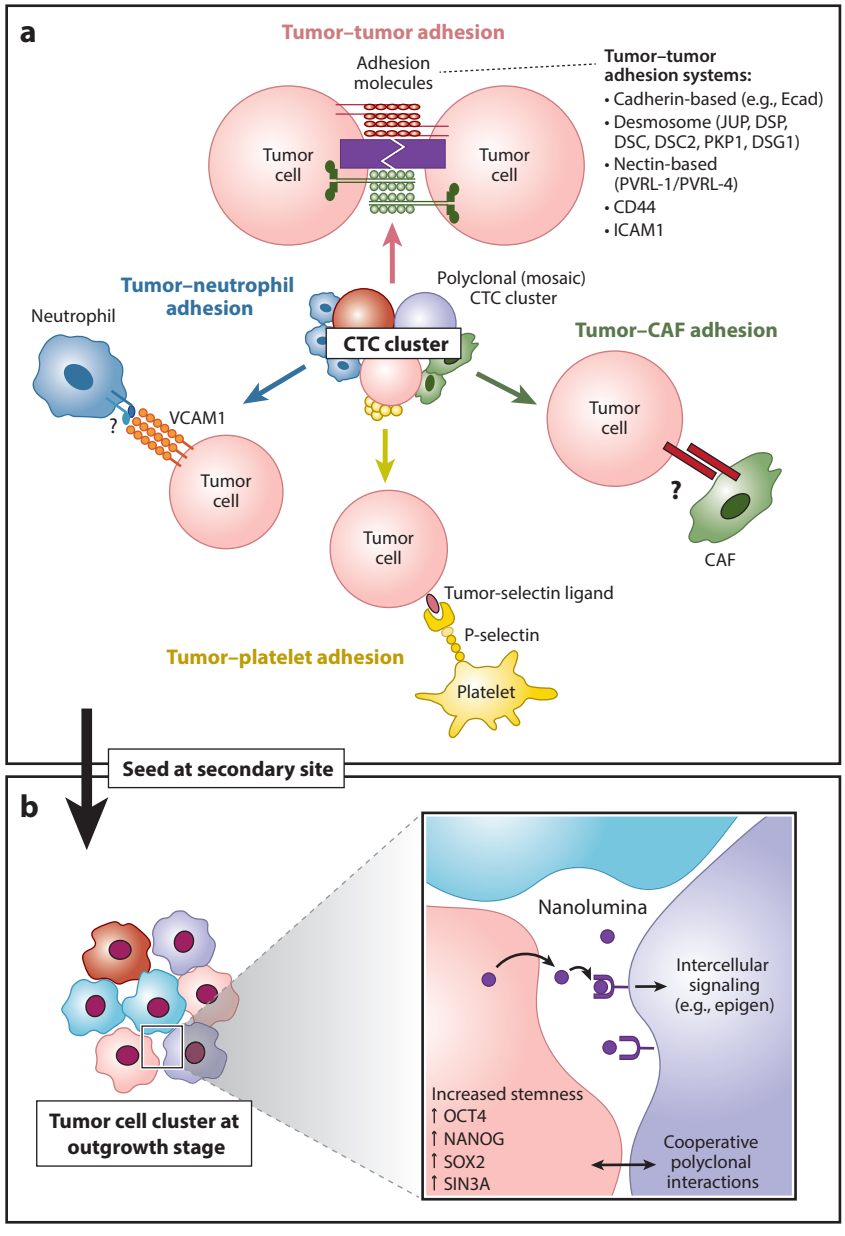

Figure 1

Homotypic and heterotypic tumor cell clusters. (*a*) CTCs adhere to each other, to neutrophils, to platelets, and/or to CAFs to form homotypic or heterotypic clusters through various adhesion mechanisms. (*b*) Once at the secondary metastatic seeding site, tumor cell clusters can have outgrowth advantages over single cells through increased stemness, intercellular signaling, and cooperative polyclonal interactions. Nanolumina, sealed intercellular cavities between tumor cells, can concentrate soluble signaling molecules for intercellular signaling. Abbreviations: CAF, cancer-associated fibroblast; CTC, circulating tumor cell; DSC, desmocollin; DSG1, desmoglein 1; DSP, desmoplakin; Ecad, E-cadherin; ICAM1, intercellular adhesion molecule 1; JUP, junction plakoglobin; OCT4, octamer-binding transcription factor 4; PKP1, plakophilin 1; PVRL, poliovirus receptor–like; SIN3A, SIN3 transcription regulator family member A; SOX2, SRY-box transcription factor 2; VCAM1, vascular cell adhesion molecule 1.

There is evidence that cell adhesion gene expression can be modified under selective pressure. For example, in vitro selection of tumor cells resistant to FSS in a microfluidic system led to a highly metastatic population with increased clustering and expression of desmosomal genes (68). Likewise, isolation of DTCs in a mouse model of head and neck squamous cell carcinoma identified clones with enhanced fitness that preferentially generated clusters resistant to anoikis when placed under FSS (83). In another example, estrogen receptor–positive breast cancers can develop resistance to endocrine therapy via genomic alterations in the estrogen receptor gene *ESR1*. *ESR1*-mutant breast cancer cells show increased expression of adhesion genes as well as enhanced clustering and metastasis (26). More work is needed to understand whether selective pressure during metastatic seeding or during cancer treatment drives the selection for tumor cells with clustering behavior.

3.2.2. Heterogeneous tumor cell populations. Tumor dissemination is a critical bottleneck that acutely restricts the diversity of tumor cell populations. In the collective cell metastasis, cells maintain the advantage of heterogeneity during invasion, in circulation, and in the metastatic niche. Once growing in the metastatic niche, these cells often give rise to diverse lineages to ensure survival (44, 84). Identification of polyclonal metastases in a variety of experimental models and clinical genomic studies supports the theory that tumor cell clusters are a vehicle for metastasis. Such phenotypic and genotypic heterogeneity is an undoubted contributor to tumor growth and therapy resistance, posing a significant challenge in the clinic (85).

Supportive of this concept, circulating clusters of melanoma tumor cells have variable expression profiles that mark INV and PRO clones, which, as described in detail below, exhibit high metastatic behavior in a multiclonal fashion (45). In another example, metastatic breast cancer CTCs have been shown to express both epithelial and mesenchymal RNAs in situ, indicating that clusters could be composed of tumor cells varying along the epithelial-to-mesenchymal transition (EMT) spectrum (86). In these cases, cells within clusters need not commit to a single state and may benefit from all stages of the EMT spectrum. Despite the presence of heterogeneous populations, many cells in CTC clusters show evidence of methylation patterns consistent with increased stem cell factors (70). In different tumor models, metastasis-initiating cells give rise to metastatic tumors that may share properties with more stem-like or regenerative cell populations (2). It remains unresolved whether tumor cells in clusters rely more on hybrid EMT or cooperation between epithelial and mesenchymal cells for successful metastasis.

In a more complex example, two breast cancer tumor cell clones expressing either IL11 or FIGF (VEGFD) cooperate with neutrophils to drive polyclonal metastasis. When IL11- and FIGF-expressing cells make up 10% of a primary tumor, metastases increase significantly. These cells signal to stromal cells and neutrophils to increase metastasis, an effect that is lost when neutrophils are depleted (74). As mentioned above, ovarian tumor cell clones expressing high levels of ERBB2 cooperate with another clone that expresses high levels of AREG during metastasis (76). These reports suggest that heterotypic tumor cell clusters are poised to take advantage of intercellular cooperation to promote seeding and metastasis.

3.2.3. Nontumor cells. Beyond tumor–tumor cell interactions, tumor cell clusters can be found in association with various stromal cells, immune cells, and blood cell components. When in concert with tumor cells, neutrophils instigate metastasis in a variety of contexts. In one report, Szczerba et al. (30) showed that while 88% of breast cancer patient CTCs were single cells, 8.5% were homotypic clusters and 3.4% were heterotypic white blood cell (WBC)–CTC clusters. Within the WBC–CTC clusters, roughly 85–91% of WBCs were neutrophils. Neutrophil-associated CTC clusters exhibited high expression of cell-cycle genes and efficient metastasis

formation. Detection of neutrophil–CTC clusters was also associated with poor prognosis in metastatic breast cancer patients when compared with patients without these clusters (30, 87). Functional screens identified that VCAM1 mediates neutrophil–CTC interactions and that VCAM1 inhibition prevents neutrophil–CTC cluster formation. This discovery suggests a potential therapy to prevent metastatically efficient neutrophil–CTC cluster formation without depleting non-CTC-associated neutrophils. Other myeloid-derived immune cells such as myeloid-derived suppressor cells (MDSCs) are known to promote neoplastic growth by inhibiting T cell activity and to form clusters with tumor cells in circulation (88). Like neutrophils, MDSCs can directly interact with CTCs and promote their dissemination and metastatic potential.

Tumor cell clusters can also be found in association with stromal elements from the primary tumor. There is evidence that the viability of CTCs is higher when they cotravel with cancer-associated fibroblasts (CAFs) as heterotypic clusters (89, 90). Tumor cell clusters in association with CAFs promote early metastatic outgrowth at the secondary site (89). These findings indicate that tumor cells can bring their own "soil" from the primary tumor with them to secondary sites of metastasis. More work is needed to determine the comparative frequency of this mechanism in tumor cell clusters compared with other modes such as reprogramming the distant tissue microenvironment.

Finally, CTC–platelet clusters promote metastasis in several ways (59, 91, 92). Labelle et al. (91) determined that platelets protect CTCs from FSS during circulation by using a viscometer to subject CTCs to prolonged periods of shear stress, modeling in vivo circulation. Egan et al. (93) similarly determined that platelets can provide protection against FSS in experiments using A2780 cells with lactate dehydrogenase release as a proxy to measure membrane damage. Platelets also grant CTCs resistance to anoikis (92). Ovarian and colorectal cancer cells cultured under anoikis conditions exhibited reduced anoikis when cocultured with platelets in a YAP1-dependent manner, and treatment of tumor-harboring mice with antiplatelet antibody increased tumor cell death (92). Similar to other immune and stromal cells, platelets can induce epithelial-mesenchymal-like transition in tumor cells (91). Platelet-tumor contact and transforming growth factor beta (TGFβ) signaling from platelets activate TGFβ/Smad and NF-κB pathways in colon and breast cancer cells. Platelet activation in turn can induce signaling changes by releasing TGFβ (91), ATP (95), or chemokines to induce EMT, transendothelial migration, and recruitment of granulocytes, monocytes, and macrophages (96). This results in an EMT-like transition to an invasive and mesenchymal-like phenotype, increasing metastatic efficiency (91). A challenge to studying the specifics of CTC–platelet cluster function in circulation, such as the adhesion mechanism, is that platelets cover CTCs so well that they mask these stealth CTCs from being detected by many traditional methods (94). Despite these limitations, in totality these studies suggest that coagulation activation of platelets generates a protective barrier against FSS and immune surveillance, inducing metastatic spread and outgrowth through tumor cell signaling and plasticity.

4. MECHANISMS OF COLLECTIVE TUMOR CELL MIGRATION AND DISSEMINATION

Many central questions in collective metastasis revolve around how tumor cell clusters migrate and disseminate into the blood and lymphatic vasculature. Intuitively, this process involves the loss of cell–cell adhesion contacts and gain of cellular motility, as commonly observed in single-cell metastasis. Here, however, some cell–cell adhesions are maintained. The migration of clusters introduces new behavioral capabilities but also introduces new challenges faced by tumor cells when orchestrating multicellular motility. As discussed below, a variety of modes have been identified by which tumor cell clusters accomplish invasion into surrounding tissues and intravasation into blood vessels (**Figure 2**).

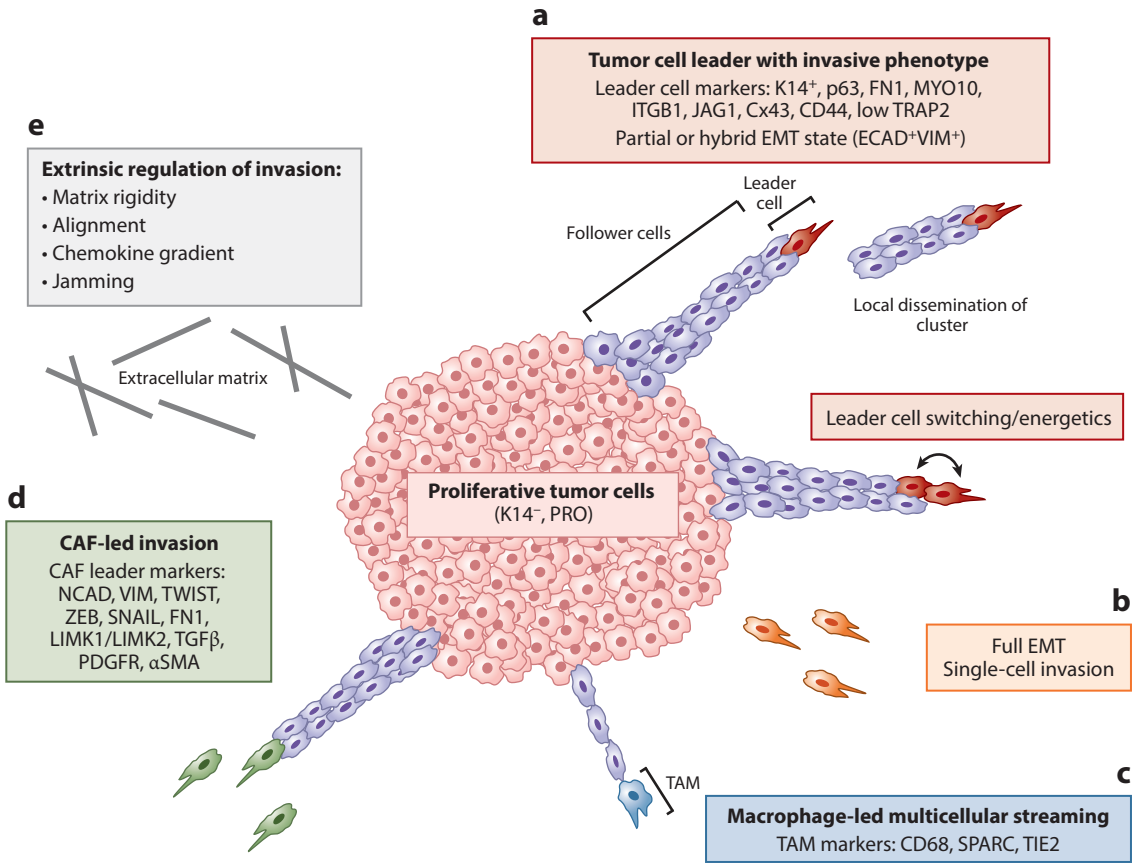

Figure 2

Migration modes in multicellular tumor invasion. Metastasis begins with the invasion of tumor cells into surrounding tissues. Diverse cancers utilize collective migration of tumor cells distinguished by heterotypic phenotypes [leader cell (*a*), partial or hybrid EMT, or stromal cells] or single-cell migration modes via complete EMT (*b*) or macrophage-directed invasion (*c*). Tumor leader cells as well as CAFs (*d*) and TAMs, which can act as leader cells, express unique markers including many MMPs and integrins (103). The relative frequency of these migration modes depends on tumor type and local microenvironmental constraints including matrix alignment, rigidity, jamming, paracrine factors, and nontumor cells within the TME (*e*). Abbreviations: αSMA, alpha smooth muscle actin; CAF, cancer-associated fibroblast; Cx43, connexin-43; Ecad, E-cadherin; EMT, epithelial-to-mesenchymal transition; FN1, fibronectin; Itgb1, integrin subunit beta 1; Jag1, jagged canonical notch ligand 1; K14, keratin-14; Limk, LIM domain kinase 1; MMP, matrix metalloproteinase; Myo10, myosin X; Ncad, N-cadherin; p63, tumor protein 63; PDGFR, platelet-derived growth factor receptor; PRO, proliferative; Snail, zinc finger protein SNAI1; Sparc, secreted protein acidic and cysteine rich; TAM, tumor-associated macrophage; TGFβ, transforming growth factor beta; Tie2, TEK receptor tyrosine kinase; TME, tumor microenvironment; TRAP2, tumor rejection antigen (Gp96) 1 pseudogene 2; Vim, vimentin; Zeb, zinc finger E-box binding homeobox.

4.1. Collective Cell Migration

In one model of collective dissemination, tumor cells collectively invade tissue surrounding the primary tumor and then enter a lymph or blood vessel as a CTC cluster. This hypothesis is supported by three observations. First, collective migration is commonly executed by cells in diverse contexts including tissue morphogenesis, wound healing, and cancer. Second, pathologists have long noted that the invasive border of human cancers is most often composed of collectively invading fronts extending from the main tumor mass rather than individually disseminated tumor

cells (97, 98). Migration of tumor cell collectives can be observed during real-time observations of tumor explants in culture and in vivo (99–102). Third, in multicolor fluorescence tracing experiments, multicolor metastases are more common when the primary tumor is composed of mixed color clones. Since multicolor metastases are rarely observed when clones are spatially separated, it is implied that clusters most commonly arise from a collective intravasation event. Together, these observations anchor a broad swathe of studies investigating the cellular and molecular mechanisms involved in collectively invading tumor cells.

4.1.1. Leader cells in collective invasion. Invading collectives can take on various morphologies, including single-cell-width strands and nonprotrusive nests. In many cases, phenotypically distinct cells termed leader cells have been identified at the foremost edge of the collective invasion front that execute a variety of migratory functions supporting collective invasion. Frequently, the ablation or functional perturbation of leader cells is sufficient to disrupt tumor invasion and metastasis (45, 100, 103, 104). In some cases, leader cells are a preexisting population within the tumor; in others, a small population of tumor cells will acquire a leader cell identity when exposed to specific microenvironmental conditions; and in still other instances, leader cells will trade positions in the setting of metabolic exhaustion (100, 105–109). These leader cell and follower states can be fixed or plastic and can express distinct transcriptional, epigenetic, or metabolic states, depending on context, that can be interrogated via marker-based sorting or via photoconvertible tagging to isolate leader- and follower-positioned tumor cells (108, 110–112). In some cases, stromal cells such as fibroblasts and macrophages take on the role of leader cell and can guide tumor cells via path clearing and activation of migratory signals (103, 113–116).

The spatial induction of leader and follower cell organization is, in many cases, associated with complementary cellular behaviors by tumor cells within clusters. These complementary behaviors are consistent with the proposal that social type interactions or intercellular cooperation between tumor cells can promote metastasis (2, 40, 85). Migratory clusters can avoid trade-offs between growth and migration through specialization; leader cells can be specialized toward matrix degradation remodeling and path generation, while nonleader cells can maintain a proliferative state (34, 45, 110, 117). In one striking example, Campbell et al. (45) examined melanoma metastasis in zebrafish and patient samples. They identified two distinct subpopulations defined as INV^{-}, which more aggressively migrate and metastasize, and PRO^{-}, which show superior proliferative capacity. INV and PRO tumor cells formed heterotypic clusters and cooperated to form polyclonal metastases. Melanoma patients harbor PRO-INV heterotypic clusters in approximately 20% of cases, supporting human disease relevance. This work, alongside others, indicates that tumor subpopulations exert complementary roles in metastatic dissemination and outgrowth (74, 100, 118).

Molecular dependencies of metastasis are sometimes leader-cell specific. For example, in a lung cancer model, blocking expression of vimentin in fibroblasts, but not tumor cells, was found to reduce local collective invasion and metastasis (116). Likewise, squamous cell carcinomas show heterotypic coupling between fibroblast N-cadherin and tumor cell E-cadherin to induce directed cell migration, and blocking E-cadherin/N-cadherin interactions is sufficient to reduce collective invasion (115). For tumors with a propensity for tumor-cell leaders, blockade of leader cells function can be sufficient to reduce metastatic dissemination. For example, inhibiting keratin-14 in a breast cancer model reduced metastases, and removing an invasive population from a melanoma model suppressed metastases in zebrafish (34, 45). At the same time, it should be acknowledged that there are different collective migrations in which there is no dedicated fixed leader (119), where follower cells may be actively propulsive and primarily drive collective migration (120), and where forms of tumor invasion are driven by nonprotrusive migrations without leader cells (36).

In one such case, colorectal cancer tumor cell clusters adopt an inverted polarity with an apical-out topology to collectively invade nonprotrusively (36). More work is needed to systematically determine which tumor cell types and migratory events do or do not involve leader cells.

4.1.2. The role of epithelial-to-mesenchymal transition in collective invasion. The regulation of collective versus single tumor cell motility is an area of major investigation. EMT is a conserved developmental program driving loss of epithelial traits and gain of mesenchymal traits that becomes co-opted to support cancer metastasis and chemotherapy resistance (1). In the single-cell model of metastasis, cells have been thought to undergo EMT during invasion, allowing for motility, and then reverse the effect through mesenchymal-to-epithelial transition upon seeding. However, in certain cancer models, complete EMT transitions appear dispensable for metastasis, only promoting chemotherapy resistance (121, 122). The dispensability of complete EMT may be explained by studies indicating that a continuum of intermediate states exists in which tumor cells take on both epithelial and mesenchymal traits simultaneously, termed partial or hybrid EMT (123). Hybrid or partial EMT states may enable tumor cells to gain enough of a motile program to acquire motility while retaining the benefits of tumor cell cohesion (103, 124, 125). Consistent with this model, dual-recombinase lineage tracing of an early and a late EMT marker uncovered that partial EMT but not full EMT cells promote collective invasion and are required for metastasis formation (122). Likewise, the EMT transcription factor Snail induces collective invasion and tumor metastasis in squamous cell carcinoma models (126) and in *Drosophila* intestinal tumor models (127). Rather than by transcriptional repression, partial EMT states could alternatively be achieved via protein relocalization and internalization of epithelial proteins (128). Collective cell motility is dependent not only on the presence of epithelial gene expression but also on the physical constraints of the local microenvironment, which can cause tumor cells with low E-cadherin to transition from single-cell to collective migration under ECM confinement (78). An unresolved question from these studies is to what extent individual and collective, epithelial and mesenchymal, migrating tumor cells outcompete each other at different steps of the metastatic process.

4.2. How Does a Tumor Cell Cluster Disseminate into the Vasculature?

Although both single CTCs and CTC clusters have been found in circulation, it is not yet well understood where, when, and how tumor cells enter blood or lymphatic vessels (129). In the traditional linear cascade model of metastasis, intravasation is depicted as a step in the metastatic cascade that occurs after local invasion at tumor–stromal borders (1). However, tumor vessels vary in diameter and permeability, which are both features that could contribute to the success rate of tumor cell dissemination. Corrosion casting of tumor vasculature has shown that blood vessels become more aberrant and less organized as they get close to the core of the tumor (130). This lack of organization may arise due to rapidly and irregularly formed vessels, resulting in leaky tumor vasculature with impaired barrier function and little lymphatic drainage (129). Tumor cells may take advantage of vessels with compromised integrity to gain access to circulation (131). In the mouse mammary tumor MCH66 model, blood vessels have been theorized to engulf nests of tumor cells, instigating metastasis without invasion (132). Alternatively, gaps in the endothelial lining, called mosaic vessels, may directly expose tumor cells to vessel flow (129). One study showed that tumor vasculature may be up to 15% mosaic (133). Recently, Silvestri and colleagues (134) developed a microvessel coculture model, which demonstrated that breast tumor organoids integrate into the endothelial cell lining and can intravasate as clusters. These findings suggest parallel processes and mechanisms that extend beyond the linear cascade sequence of metastasis. For example, in a chick chorioallantoic membrane mesoderm model, Deryugina & Kiosses (135) showed that intravasation can begin early in tumor progression and proceed in parallel with

MANEUVERING THROUGH SMALL VESSELS

The time spent in circulation by CTC clusters is shorter than that of individual tumor cells (17), presumably because CTC clusters become trapped in proximal vessels. However, microfluidic demonstrations show that tumor cell clusters display remarkable flexibility. Tumor cell clusters up to 20 cells in number can reversibly reorganize into single-file chains in capillary-sized vessels in microfluidic devices and in zebrafish models (141). Weakening of intercellular adhesions resulted in dissociation of clusters into single cells, while strengthening of intercellular adhesion resulted in occlusion of clusters in the constrictions, indicating that adhesion composition could dictate circulatory arrest. More work is needed to establish how the interplay of mechanical stress and cell–cell adhesion in CTC clusters impacts their metastatic potential in vivo and in cancer patients.

local stromal invasion. Interestingly, they also showed that most intravasation events occur in the interior region of the primary tumor, not the tumor–stromal border. Likewise, breast tumor xenograft models demonstrate the existence of hypoxic cores that induce intravasation of CTC clusters that were largely hypoxic, whereas individual CTCs were largely normoxic (136). The development of more realistic and robust models to study tumor cell cluster intravasation events, which are very rare, is needed to better define the mechanisms by which tumor cell clusters into the circulation.

Another possibility is that tumor cells disseminate individually and then aggregate within the local circulation before being detected as CTC clusters. Consistent with this possibility, different breast cancer models have supported early dissemination of individual cancer cells of the $Her2^{+}$ subtype and implicated involvement of TEK receptor tyrosine kinase ($Tie2^{+}$) macrophages (137, 138). In these cases, Tie2-high, vascular endothelial growth factor A (VEGFA)-high macrophages induce transient vascular permeability, invadopodia formation, and intravasation of actin-regulatory protein mammalian-enabled (MENA)-high tumor cells into adjacent endothelial cells (139, 140). Furthermore, recent studies have suggested that tumor cells that have undergone intravasation subsequently aggregate in blood vessels via CD44-dependent homophilic adhesion to seed metastasis as polyclonal clusters (38). It seems likely that the relative proportion of seeding events that are cohesive versus due to aggregation will depend on the local density and spatial proximity of tumor cells, which in turn will depend on the abundance of tumor cell dissemination or the number of tumor cells injected intravenously in the case of experimental metastasis assays. At present, our understanding of the extent to which tumor cell clusters intravasate through a different mechanism or a similar mechanism to single tumor cells remains poorly characterized and will benefit from new experimental models and clinical samples focused on capturing tumor intravasation events. However, as discussed in the sidebar titled Maneuvering Through Small Vessels, work has been done to show that bulky tumor cell clusters can travel through small vessels together.

5. CANCER TREATMENT FROM A COLLECTIVE METASTASIS PERSPECTIVE AND FUTURE OUTLOOK

The ultimate goal of cancer research is to develop therapies and diagnostic tools that will eradicate and prevent cancer. While the collective metastasis of tumor cell clusters to distant sites remains a field in relative infancy, several major principles emerge, as discussed below.

For multiple phases of metastasis, tumor cells undergo a major boost when they are in physically cohesive clusters. These properties are apparent not only during dissemination (in transit) where tumor cell clusters are better shielded from physical and immunologic insults but also

after arrival in the distant organ, supporting advantages for multicellular cohesion in survival, proliferation, and outgrowth into overt metastases. These robust effects are mediated in turn by adhesion systems, signaling molecules, and the spatial architecture of clusters that facilitate cooperation between tumor cells and are further supported by the actions of different assemblies of phenotypically distinct tumor cells and host cells. Despite the revolution in immunologic targeting and targeting of the tumor microenvironment, the core armamentarium of cancer therapy (cytotoxic chemotherapy, biologics, and small molecules) remains directed toward targeting cell-autonomous intracellular processes. Beyond these approaches, collective metastasis suggests the general principle of targeting intercellular tumor interactions as a strategy for anticancer therapy.

5.1. Targeting Adhesion

Targeting tumor cell cluster adhesion has been proposed as a means of antimetastatic therapy. However, targeting adhesions could also be problematic; for example, epithelial cells in the major organs also harbor adhesion complexes including adherens junctions and desmosomes, and therefore greater specificity for tumor cell cluster adhesion will be needed for an adequate therapeutic index. In addition, disrupting cell–cell cohesion could induce counterproductive increases in tumor dissemination or disrupt clusters into smaller aggregates, which could increase rather than decrease metastatic seeding. Thus, the consequences of wholesale targeting of cell adhesion remains unclear. Another approach is to target adhesion and adhesion signaling between tumor cells and nontumor cells in clusters such as neutrophils and platelets. For example, integrin adhesion-targeted therapy against platelets is highly effective for the treatment of acute myocardial infarction. Tumor cell clusters show methylation patterns consistent with greater stemness factor expression (30). Targeting of stemness factors such as CD44 could reduce tumor cell cluster adhesion as well as impair stem cell–associated signaling involved in early metastasis formation (38).

5.2. Targeting Collective Signaling

Another approach is to focus on the collective intercellular signals induced in tumor cell clusters. For example, some clusters utilize intercellular EGFR family receptor signaling in homotypic and heterotypic clusters, and blocking these signaling mechanisms can markedly reduce metastasis in animal models (39, 76). Thus, blockade of collective intercellular signaling, and the specific receptor repertoires at intercellular contacts of tumor cell clusters, could offer a means to target micrometastatic disease and therapy-resistant tumor cells. It is unclear whether cluster-induced signaling is a property only of small clusters, but the evidence of intercellular signaling in macrometastases suggests that these collective intercellular signals persist through development of overt metastasis. In this case, targeting collective signaling could have benefits for both micrometastatic and macroscopic metastasis.

5.3. Targeting Heterogeneity

A third approach is to target the interacting tumor cells within clusters themselves, which take on distinct cellular roles such as migration and proliferation and express distinct surface repertoires. Heterogeneity of marker expression will reduce the efficacy of cell-specific, cell-targeted antibodies. For example, $HER2^{+}$ breast cancers that are heterogeneous for HER2 expression show worse responses to HER2-targeted antibody therapy (142), while HER2-antibody drug conjugates that release their cargo both on the target cell and on neighboring cells have been proposed to more effectively target HER2-low or heterogeneous breast cancers. A challenge is

the significant plasticity shown by many tumor cells, leading to interconversion events between different states. Inhibition of plasticity or, alternatively, combinatorial targeting of multiple cell populations could, in principle, yield more durable responses to therapy.

5.4. Summary

Whether it is targeting clusters for destruction, preventing them from forming, preventing them from entering circulation, or inhibiting their augmented growth, a key challenge is determining which cancer patients would stand to gain from therapies targeting collective interactions between cells. One possibility is that these intercellular interactions are most important for dissemination and outgrowth but become dispensable beyond a certain point. In this case, anticluster therapy will need to be paired with other cytotoxic therapies that do have efficacy in macroscopic disease in the metastatic setting. However, another possibility that we think is likely is that at least some of these intercellular collective interactions persist from the microscopic cluster to the overt metastasis stage. Indeed, therapeutic targeting of cell adhesion molecules expressed on metastases such as Trop2 and nectin-4 has had robust effects in clinical trials in patients with advanced metastatic disease (143, 144). In this case, the study of tumor cell clusters provides a clue for the core dependencies that emerge early in metastasis formation. The prediction here is that collective metastasis–targeted therapy should eradicate macroscopic and microscopic tumors.

An additional consideration while targeting collective metastasis is the importance of host factors that may predispose cancers toward clustered dissemination and collective metastasis. For example, inflammatory breast cancers are prone to forming tumor emboli highly expressing E-cadherin with aggressive metastatic trajectories. Clinical studies collected from larger patient populations will be necessary to design informed therapies targeting collectivity. We know that the presence of CTC clusters in patients' blood is an indicator of poor prognosis compared with patients with only single CTCs. However, there is not much known about predictive markers that anticipate CTC cluster dissemination and collective metastasis. Are there some patients who are more prone to developing collective metastases based on factors such as genetics, environmental factors, and therapy exposure? This is an important area of study that is highly understudied but could improve therapeutic strategies and outcomes, and there is virtually no information about how patient demographics relate to collective metastasis.

6. OUTLOOK

Through recent studies, the salient features of collective metastasis are emerging. Key observations include that CTCs occur both as single cells and as aggregated clusters, that these CTC clusters are potent seeds for new metastases, and that these CTC clusters generate polyclonal metastases in animal models. An attractive hypothesis is that CTC clusters deliver genetic and phenotypic heterogeneous tumor (and in some cases stromal) populations that promote metastasis and therapy resistance. At the same time, CTC clusters have now been shown to encompass a variety of properties considered useful for metastasis—including enhanced survival, anoikis resistance, immunoevasion, collective signaling, and outgrowth. We anticipate that as the field grows, a clearer picture may emerge for the major molecular components involved in collective metastatic seeding, providing a road map for cancer therapeutics.

There continues to be debate about what instigates the collective migration of tumor cell clusters and how these clusters disseminate into circulation. At present, there is strong evidence for collective invasion of tumor cells, cooperative interactions between different cell types, potentially social-level behaviors such as resource sharing, and division of roles within the cluster; there is also robust evidence that heterotypic cell populations play important roles in supporting different

stages of metastasis. There is significant work showing that partial or hybrid phenotypic EMT states may contribute to tumor dissemination and, because these cells do not lose their epithelial character completely, show preference for collective migration. Whether cooperative cell populations or cell plasticity toward intermediate states are most important for the initiation of collective migratory behavior is not resolved, and indeed both processes could be operative simultaneously.

Most importantly, the timing and site of dissemination, and whether there are differences between the dissemination of single cells and clusters, are presently unknown. On the one hand, there is a tendency for CTC clusters to co-occur with a high abundance of CTCs. On the other hand, single tumor cells and collectively migrating cells would seem to require distinct cellular programs given their physical constraints. It remains to be tested whether there is a common driver of CTC and CTC cluster dissemination or whether there are favored mechanisms for the aggregation or disaggregation of CTCs into either form. We expect that improvements in technology to identify these rare events and models for collective dissemination will help to answer these questions.

Finally, a key challenge is to bridge the gap between these basic studies of collective metastasis and clinical application for therapeutic targeting of metastasis. We suggest that collective interactions between cells, whether of the same or different phenotype and therefore the same or different surface repertoire, are likely to be important at all stages of metastasis. We expect that a concerted effort to define the intercellular interactions driving the collective migration and dissemination of clusters of cells will therefore be highly promising to unveil new paradigms for metastasis research and cancer treatment.

DISCLOSURE STATEMENT

K.J.C. is an inventor on a pending patent application related to collective signaling.

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