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Metastasis inside-out: dissemination of cancer cell clusters with inverted polarity

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Zajac *et al* (2018) utilize patient tissue to discover a novel cellular mechanism for metastasis in colorectal cancer. They isolated cancer cell clusters from patient peritoneal fluid and demonstrated that these clusters invaded collectively into collagen gels, patient-derived tissues, and through the peritoneum of mice. During invasion the clusters retained an inverted epithelial architecture, functioning as multicellular metastatic seeds with inverted polarity.

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See also: **O Zajac *et al***

We teach students that cells invade into surrounding tissues via molecular specializations on their basal surface and that metastases are seeded by single cells. A recent study from Zajac *et al* (2018) provides compelling evidence that mucinous colorectal cancers do not attend our lectures and instead metastasize to the peritoneum as large epithelial clusters with an inside-out organization, invading into the ECM with their apical surface.

Understanding the mechanisms driving metastatic spread is important, as it causes the majority of cancer deaths (Siegel *et al*, 2016). Metastasis remains incompletely understood in part due to the slow nature of progression, its location deep inside the body, and its involvement with the systemic biology of the organism. It has been difficult to model adequately in the laboratory or to observe its intermediate steps in *in vivo* systems, though progress is being made (Alexander *et al*, 2013; Harney *et al*, 2015). Accordingly, conceptual paradigms for metastasis have typically relied considerably on inference. The dominant model in the literature has been the epithelial-to-mesenchymal transition (EMT), which posits that cancer cells partially or completely lose their epithelial properties, detach and travel as single cells, and form clonal metastases (Kalluri & Weinberg, 2009). An alternate theory has arisen, of collective epithelial metastasis, whereby groups of cancer cells invade and disseminate collectively to found multiclonal metastases (Aceto *et al*, 2014; Maddipati & Stanger, 2015; Cheung & Ewald, 2016; Cheung *et al*, 2016). Understandably, this literature has relied heavily on the use of human cancer cell lines and transgenic mouse models, each of which has strengths and limitations. The present study instead relies exclusively on colorectal cancer patient tumor tissue and associated patient-derived xenograft models. Their goal was to determine how colorectal cancer (CRC) seeds peritoneal metastases.

Detecting potential metastatic seeds in patients

The study launches from an examination of cells recovered from the peritoneal effusion of patients with CpG island methylator phenotype (CIMP) colorectal cancer, a subtype known to associate with a high rate of metastatic recurrence. This fluid contained cancer cell clusters and single cancer cells. They next sought to determine the relative importance of clusters and single cells. The lineage analyses and functional *in vivo* experiments that have enabled the retrospective analysis of the single-cell vs. cluster origins of metastases in model organisms (Aceto *et al*, 2014; Maddipati & Stanger, 2015; Cheung *et al*, 2016) are not possible in human patients. The authors overcame this challenge through a rigorous series of experiments aimed at determining the abundance and functional properties of different potential metastatic seeds.

Evidence that mucinous CRC spreads via a multicellular seed

They first assayed the contents of peritoneal effusions from 43 patients and found clusters at 66 times the frequency of single cells. To avoid enrichment for epithelial phenotype cells by positive selection (e.g., for EpCam), they analyzed total cellular fractions or the total following negative selection for $CD45^+$ cells. They then quantified cluster frequency across colorectal cancer subtype and in relation to the presence of peritoneal metastases. They found that clusters were most frequent in subtypes associated with poor patient outcomes, such as mucinous, micropapillary, and cribriform. There was also a striking correlation between the absence of peritoneal metastases and the absence of clusters in the peritoneal effusion. The authors conclude that the clusters are in the right places at the right times to seed metastases.

They next sought to infer whether it was likely that the clusters travel in groups or whether they travel as single cells and aggregate at distant sites, through processes such as a mesenchymal-to-epithelial transition (MET). They first assayed for EMT molecular markers in the clusters and did not find enrichment in these markers or morphological evidence for EMT or MET processes. However, their methods cannot exclude the existence or contribution of rare populations of EMT-like cancer cells to metastasis in this system. They next tested the capacity of single cells isolated from peritoneal effusions to form clusters or colonies, relative to clusters

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isolated from the same patient. Essentially all of the single cells died, whether cultured in suspension or in ECM. This observation is consistent with the very limited ability of single breast cancer cells to form colonies in culture or in tail vein injection assays (Aceto *et al*, 2014; Cheung *et al*, 2016). Cluster organization, rather than some cell intrinsic property of the clustered cells, explains this survival difference, as single cells isolated from disaggregated clusters also die rapidly. The converse was also true: Induced aggregation promoted survival.

Cellular and molecular features of the metastatic seed

The first striking feature of these clusters is that they are large, containing an average of 257 cancer cells (Fig 1). Second, they retained both molecular markers ($EpCam^+$, $K20^+$, Vim^-) and ultrastructural features of epithelial differentiation. However, they were organized in an inverted configuration, with apical markers facing the extracellular matrix (ECM). These characteristics led the authors to term the clusters tumor spheres with inverted polarity (TSIPs). They next demonstrated that TSIP-like structures could be identified across multiple different stages of invasion and metastatic spread in patient tissue samples, lending further plausibility to their central role in dissemination. This histological examination led the authors to propose that TSIPS form by collective apical budding into the lumens of glandular structures; disruption of gland structure then enabled TSIPs to access the connective tissue and eventually the peritoneum. They observe that TGFβ signaling through SMAD2 regulates the production of TSIPs through collective apical budding. Conversely, inhibiting ROCK or myosin II pharmacologically decreased the production of clusters (Fig 1).

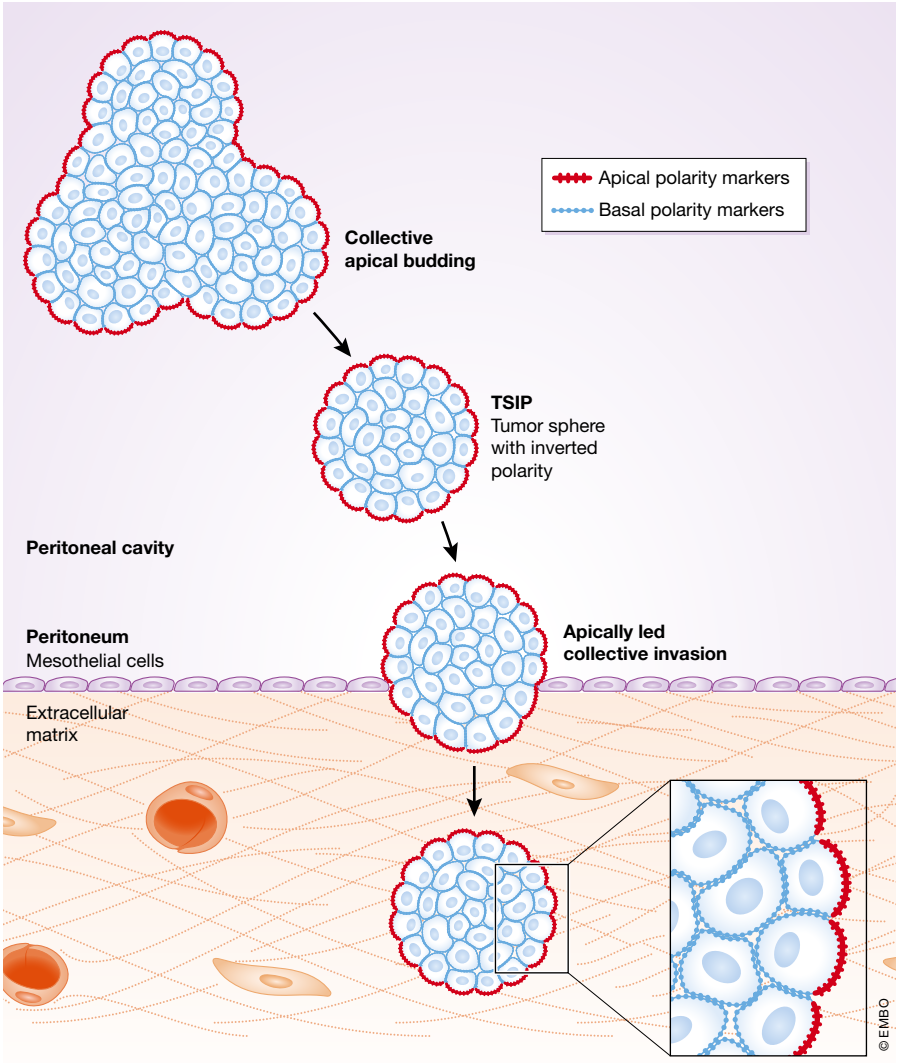

Figure 1. Dissemination and invasion of tumor spheres with inverted polarity (TSIPs).
TSIPs are generated by collective apical budding, both in the primary tumor and in the peritoneal fluid. These resulting TSIPs consist of hundreds of cells in an inverted configuration, with apical polarity markers on the external surface and basal polarity markers localized to cell–cell contacts. Surprisingly, TSIPs maintain this inverted configuration as they invade into the peritoneum and through the underlying extracellular matrix. This apically led collective invasion requires Rho and actomyosin contractility but Fak, Rac, and integrins were dispensable.

Molecular requirements for apically led collective invasion

Their data suggested that clusters could invade collectively, despite their inverted configuration. To test this hypothesis, they isolated peritoneal tissues from CRC patients and cultured TSIPs on their surface. Strikingly, the clusters invaded with their apical surface oriented toward the ECM, while single cells failed to invade. Apically led collective invasion was slow but in accordance with the timing in patients. Consistent with the fact that the basal surface was not in contact with ECM, invasion and migration of clusters were independent of FAK, Rac, and integrins. Invasion did however require actomyosin contractility and clusters displayed enriched phospho-myosin at the ECM facing surface. This myosin-dependent, integrin-independent mode of invasion in TSIPs is reminiscent of amoeboid single-cell migration patterns. They then tested the metastatic capacity of single cells vs. clusters following intraperitoneal injection into mice and observed a large increase in metastasis in clusters relative to single cells, with invasion into multiple adjacent organs as $EpCam^+$, Vim^- masses.

Conclusions and implications of metastasis by TSIPs

TSIPs are more abundant, more adapted to survival and colony formation in fluid and ECM environments, are capable of invading patient-derived peritoneal tissue, and can metastasize efficiently in mice. They are therefore highly plausible as the main or

exclusive metastatic seed in this subtype of colorectal cancer. However, the study design cannot exclude the possibility that another mechanism, such as EMT, is also contributing to metastatic spread. Their multicellular nature suggests that TSIPs could contain multiple genetic clones, each with varying fitness in different environments, different roles in the metastatic process, and differential drug resistance. Patient outcomes could be dominated by the fastest growing or most drug-resistant metastases, rather than the average TSIP. Accordingly, heterogeneous clusters could prove disproportionately important.

The most surprising result in the study is the polarity inversion of the TSIPs. There is precedence that apical basal polarity of epithelial cysts can be rapidly reversed, for example, by addition of phosphoinositides (Martín-Belmonte *et al*, 2007). There is also evidence that epithelial cells initially polarize with apical determinants on the ECM facing surface and then actively endocytose these components to generate an apical compartment (Bryant *et al*, 2014). However, apically led collective invasion of ECM was not, to my knowledge, anticipated. It is nonetheless supported by their morphological and functional data in collagen I gels, in peritoneal explants, and during dissemination into the peritoneum in patients. The authors propose that this is the first report of a collective analogue of the propulsive amoeboid migration style observed in single cells migrating through 3D matrices.

Future directions and open questions

The present study highlights the urgent need for discovery science in cancer biology. We do not yet have a complete understanding of the types of metastatic processes occurring in human patients, let alone their relative abundance across organ sites and disease subtypes. It also provides further support for the concept of epithelial paths to metastatic spread (Cheung & Ewald, 2016).

Important open questions remain. What is the contribution of stromal cells to TSIP generation and dissemination? How does apical membrane-led invasion work mechanistically? Is polarity inversion maintained throughout metastatic outgrowth or does it revert as the metastasis expands? In closing, it is clear that there are multiple mechanisms for metastatic spread. Our challenge going forward is not to prove the universality of a specific mechanism but rather to (i) determine which mechanisms are utilized by which cancers and (ii) develop therapeutic strategies to block the formation and treat the emergence of metastatic lesions by whatever mechanism they arise.

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