


The gate to metastasis: key players in cancer cell intravasation

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Metastasis is a leading cause of cancer-related death and consists of a sequence of events including tumor expansion, intravasation of cancer cells into the circulation, survival in the bloodstream, extravasation at distant sites, and subsequent organ colonization. Particularly, intravasation is a process whereby cancer cells transverse the endothelium and leave the primary tumor site, pioneering the metastatic cascade. The identification of those mechanisms that trigger the entry of cancer cells into the bloodstream may reveal fundamentally novel ways to block metastasis at its start. Multiple factors have been implicated in cancer progression, yet, signals that unequivocally provoke the detachment of cancer cells from the primary tumor are still under investigation. Here, we discuss the role of intrinsic properties of cancer cells, tumor microenvironment, and mechanical cues in the intravasation process, outlining studies that suggest the involvement of various factors and highlighting current understanding and open questions in the field.

Introduction

Metastasis is the leading cause of cancer-related deaths [1]. It proceeds via a sequence of events, beginning with tumor expansion and progressing with the intravasation of cancer cells into the circulation, their subsequent survival within the blood stream, extravasation at distant sites, and finally metastatic colonization and expansion. Each of these steps is considered to be extremely inefficient, estimating that < 1% of cells that intravasate into the circulation ultimately succeed to establish a distant metastasis [2]. However, tumors are composed of billions of cells and, as a consequence, it is assumed that millions of individual cells may be shed into circulation from 1 g of cancerous tissue in rodents each day [3]. Whether intravasation is a passive or actively regulated phenomenon is debated,

yet, unarguably, the identification of those mechanisms that trigger the entry of cancer cells into the bloodstream may reveal fundamentally novel ways to block metastasis right where it begins (Fig. 1).

Intravasation is a process whereby cancer cells detach from the tumor mass and penetrate through the endothelial walls of blood vessels to reach the blood stream, where they are referred to as circulating tumor cells (CTCs). Intravital microscopy studies have shown that cancer cells generate protrusions that are first aligned along endothelial cell–cell contacts and then inserted in between endothelial cells [4,5]. Both single cells and clusters of cancer cells together with partnering cells from the microenvironment can intravasate into blood [6–10], with cluster presence in the

Abbreviations

CAF, cancer-associated fibroblast; CTC, circulating tumor cell; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; i.v., intravenous injection assay; IFP, interstitial fluid pressure; Mena, actin-regulatory protein mammalian enabled; mtDNA, mitochondrial DNA; NETs, neutrophil extracellular traps; ROS, reactive oxygen species; TAM, tumor-associated macrophage; WES, whole exome sequencing.

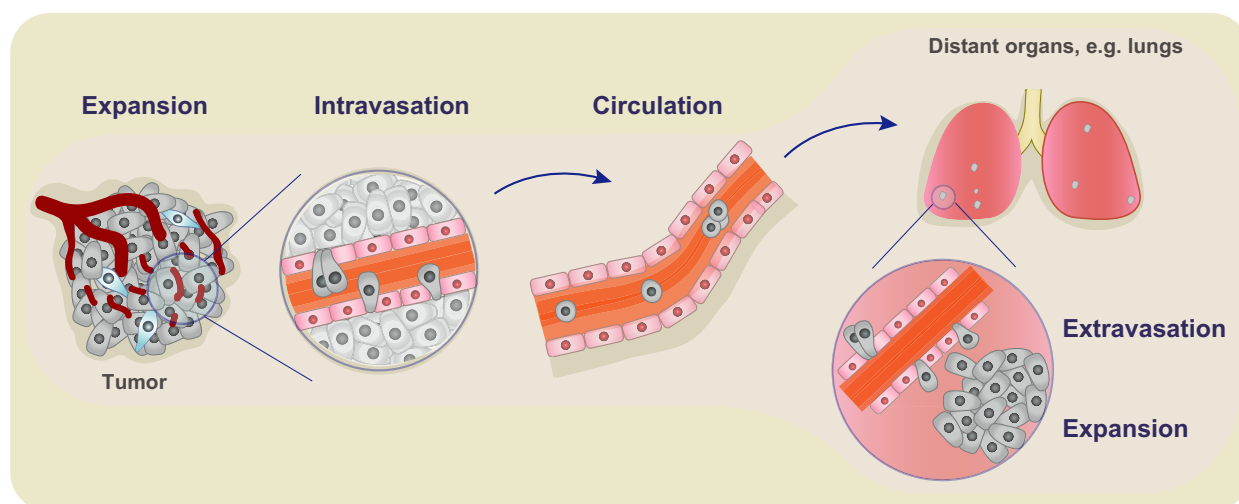


Fig. 1. Metastasis consists of a series of steps including tumor expansion, intravasation of cancer cells into the circulation, their survival in the circulation, followed by extravasation and expansion at distant sites.

circulation associated with a more aggressive disease progression [6,7].

It has been discussed whether the process of intravasation of cancer cells and shedding of CTCs into circulation is occurring randomly or is predetermined by specific factors. While no definitive proof is present at the moment, and likely more than one mechanism is involved, it is hard to imagine that intravasation would be neutral with respect to all the environmental and intrinsic factors such as proximity to blood vessels, mechanical forces, presence of immune cells, or the aggressiveness of a particular subclone of tumor cells (Fig. 2). To verify this, there is a need for rigorous studies that would prove causality between different factors and intravasation.

Multiple elements have been implicated in cancer progression in general, ranging from genetic, transcriptional, immune cell involvement, and others [11]. However, it is a challenge to disentangle the processes that directly contribute to individual steps of the metastatic cascade, given the complexity of growing tumors and their associated microenvironments. With that regard, the goal of this review is to outline those studies that suggest a linkage of various factors with the intravasation process itself, highlighting the current understanding and open questions for metastasis research. These factors include intrinsic properties of cancer cells, signals from the tumor microenvironment, and mechanical cues.

Intrinsic properties of cancer cells

Cell-intrinsic properties may play a key role in promoting intravasation of cells into the circulation.

Particularly, here we discuss how genetic mutations, gene expression profiles, epigenetic profiles, and metabolism of cells may affect their proclivity to intravasate. These factors have been discussed to be essential in various aspects of tumor development and progression. However, much information comes from correlative studies that analyze tumor profiles and disease outcome or, alternatively, that focus on the overall disease outcome without distinction into individual steps of the metastatic cascade. A useful approach to dissect the role of various factors in the intravasation process is to focus on CTCs—given their short half-life in circulation [6,12]—considering them as a ‘snapshot’ image of those features that cancer cells have acquired in order to leave the tumor site, for example, when compared to cells that did not intravasate.

Gene expression profiles

Though the expression of several genes has been implicated in cancer metastasis [13–16], their role in specific steps of the metastatic cascade remains to be fully understood. The expression of some genes may predispose cancer cells to intravasate into circulation: for instance, the role of certain signaling pathways has been emphasized in this context, in particular the Wnt pathway and epithelial-to-mesenchymal transition (EMT) program. The expression of genes involved in Wnt signaling is enriched in pancreatic cancer mouse model CTCs [17], and a study using mouse orthotopic models of glioblastoma and CTC samples from glioblastoma patients has suggested that Wnt promotes the expression of stemness markers such as

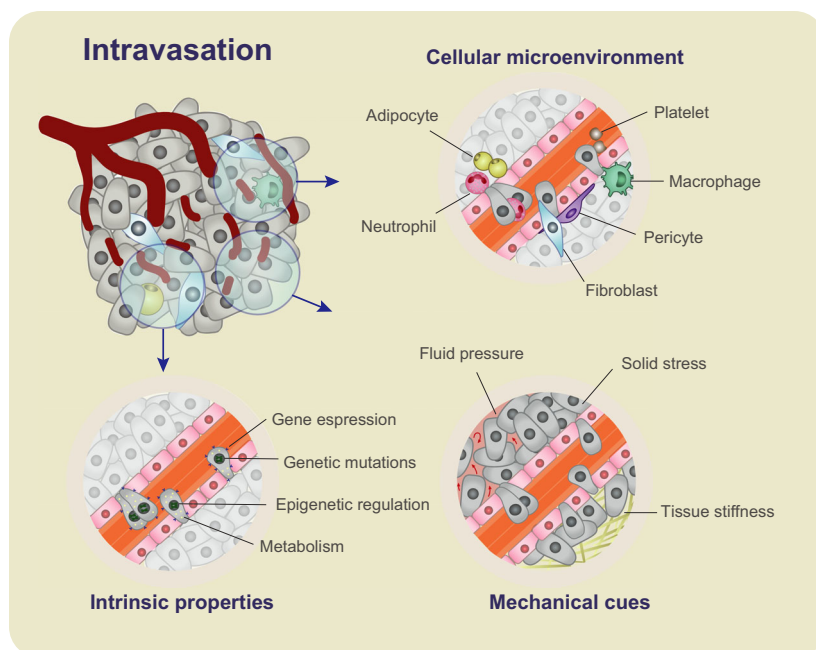


Fig. 2. The intravasation process is regulated by intrinsic, microenvironmental, and mechanical factors. Intrinsic properties include genetic mutations, epigenetic changes, gene expression, and metabolic profile alterations that give cells an intravasation advantage. The cellular microenvironment consists of fibroblasts, adipocytes, pericytes, platelets, and immune cells such as macrophages and neutrophils. These cells may regulate the intravasation of cancer cells by cell-to-cell signaling, altering the tumor microenvironment and direct participation in the invasion process. Mechanical cues such as tissue stiffness, stress, and interstitial fluid pressure may also affect cancer cell dissemination into the circulation.

Sox2, Oct4, and Nanog, with upregulated levels in CTCs compared with the primary tumor counterpart. Functionally, those cells showed enhanced proliferation and contribution to tumor growth *in vivo* [18]. Recently, in an effort to group and unify the sequencing data in relation to metastasis in patients, a database of published CTC and primary tumor RNA expression profiles has been generated [19]. Furthermore, a study analyzing comparative expression of genes between primary and secondary tumors in patients identified the involvement of genes including Wnt8A, Fgf8, pik3CB, ESR2, and others, as well as ORH5221, CATSPER4, and CLRN1 that were not previously known in the context of breast cancer [20]. ‘Stemness’ genes, in turn, were implicated in CTCs of PDAC—Lin28B and Klf4, as well as Wnt5a, and LGALS3. Lin28B knockout resulted in a reduced cell aggressiveness *in vitro* and *in vivo* [21]. Altogether, the Wnt pathway emerges as a strong candidate in cancer invasiveness, since its components appear to be over-represented in CTCs. However, more studies will be needed in this area to fully dissect the gene expression changes of CTCs compared with primary tumor cells in various cancer types, and beyond correlative efforts, to conclusively demonstrate a functional role for specific genes in dictating intravasation dynamics.

Epithelial-to-mesenchymal transition has been implicated in cancer progression [11] and is a process whereby junctions of cells are disrupted, epithelial-like cells acquire a mesenchymal phenotype, resulting in

invasive properties and detachment of single cells. While EMT has an effect on invasive properties *in vitro* and *in vivo*, it is not fully understood whether it plays a role in the spontaneous intravasation of cancer cells into the circulation and, as a consequence, enhanced colonization of distant organs [22,23]. In mouse models, the ectopic activation of EMT transcription factors induced invasive phenotype and intravasation into circulation [24–26], although contrary reports, whereby more epithelial cells were migratory, also exist [27,28]. In studies without overexpression systems, more complex hybrid phenotypes have been observed [29,30], arguing in favor of cellular plasticity rather than a full, rigid EMT transition. Some of the conclusions from the analysis of EMT mouse models may be in conflict with the data from patient tumor histology; for example, primary breast tumors are overwhelmingly epithelial [31]. Recently, a study showed that in breast cancer xenograft models, E-cadherin expression is essential for metastasis formation [32]. On the other side, E-cadherin negative cells have been seen shown to be more migratory in an intravital study of aggressive breast cancer mouse model; however, intravasation events themselves were not recorded [33]. In patient samples, CTCs with a broad spectrum of EMT features have been detected, although some of these studies used microfluidic enrichment without unequivocal validation of their putative cancer identity, for example, by means of genomic analysis [34,35]. A recent study, on

the other hand, found that there was no correlation between EMT and the clinical stage of hepatocellular carcinoma [36]. At the same time, collective cell migration and detection of CTC clusters in the circulation with evident epithelial features has been reported [6–8,10,32]. Altogether, while occurring at different degrees during cancer progression and in a model-dependent fashion, recent studies argue that EMT may be dispensable for intravasation in human disease. In our opinion, more focus should be paid to cellular programs and plasticity, and should be studied out of the box of EMT dogma, though not necessarily excluding it.

Genetic mutations

Similarly to gene expression profiles, it is conceivable that the acquisition of a particular set of mutations may confer a high propensity to intravasate, consistent with the emergence of mutationally defined, metastasis-prone tumor subclones. A few studies have investigated the mutational profile of CTCs compared with primary tumor cells in patients and identified private mutations specific to CTCs [37–41]. In prostate cancer, whole exome sequencing (WES) of matched tumor and CTC samples from two patients and subsequent SNV analysis revealed mutations in DNAH8 encoding a dynein heavy chain and in receptor tyrosine kinase EPHB1 [37]. Another WES analysis revealed genes involved in cytoskeleton modeling (e.g., MACF1) or invasion (NEDD9) as exclusively mutated in prostate cancer CTCs [39]. In breast cancer, loss of chromosome 1p was observed in CTCs and absent in the primary tumor counterpart [40]. However, it is important to note that some of these CTC-only mutations may emerge due to low depth of sequencing of the tumor or its incomplete sampling and may actually still be present subclonally within primary tumor cells [42]. Of note, several studies have identified genes promoting metastasis to defined organs [14–16]; however, the associated profiles seem reflective of the adaptation of cells to specific environments following extravasation. Nevertheless, some genes such as Nedd9, which was identified as a melanoma metastasis gene [13], have been found privately mutated in CTCs relative to tumor [39]. A higher depth of sequencing combined with an increase in the number of sampled CTCs could potentially reveal more genes simultaneously mutated or expressed in CTCs and metastases. Together, while it is likely that certain mutations may increase CTCs' proclivity to intravasate, additional sequencing efforts and functional studies will

be needed for a better understanding of this phenomenon.

Epigenetic factors

DNA methylation, histone modifications, and chromatin remodeling have been characterized in various tumors [43,44], yet the effect of those epigenetic changes on metastasis are only beginning to be resolved.

Comparative analysis of the methylation landscape of mouse primary tumor and metastatic cells [45], as well as of primary tumor and metastasis patient-derived cell lines [46], has indicated epigenetic factors that may drive metastasis. Focusing on CTCs, the hypomethylation of tumor suppressor and metastasis suppressor genes including CST6, Sox17, and BRMS1 have been observed in breast cancer patients [47]. Recently, a genome-wide methylation profile was reported for CTCs in breast cancer patients and mouse models [48]. The study revealed differential methylation pattern between single and clustered CTCs, with CTC clusters showing DNA hypomethylation at binding sites of transcription factors that are typically involved in the regulation of pluripotency and stemness in embryonic stem cells, such as OCT4, NANOG, SOX2, and SIN3A. Analysis of The Cancer Genome Atlas revealed that the hypomethylation pattern of CTC clusters was also detected in primary tumors and correlated with a poor prognosis of patients [48]. This highlights the possibility that epigenetic signatures present at the primary tumor level may predispose toward intravasation. Further comparative analysis of epigenetic patterns in CTCs and primary tumor samples may help to identify epigenetic marks with an impact on cancer cells intravasation.

Outlook

Molecular analysis of single and clustered CTCs and their comparison with primary tumor cells has begun to highlight some of the cell-intrinsic factors that could play a role in the intravasation. These include metastasis-promoting shifts in gene expression, acquisition of mutational profiles, and epigenetic patterns that favor the metastatic process. A major challenge in this regard is to compare these profiles to an adequate number of matched primary tumor cells and to minimize technical biases related to sample characteristics (e.g., solid vs liquid biopsy) and procedures (e.g., single cell vs bulk sequencing). It will also be important to focus these analyses on a high number of patient samples, that is, progressively stepping away from

model systems that may not fully recapitulate the complexity of the intravasation process in cancer patients. Integration of multi-omics methods along with dedicated computational pipelines will be instrumental in this context, promoting the identification of cell-intrinsic features with an impact on the intravasation process.

Metabolism of cancer cells

Metabolism emerges as an uneven characteristic of tumor cells with a potential impact on various steps of tumor progression, as cancer cells display a significant extent of metabolic plasticity and heterogeneity. They can rewire their metabolic profiles to ensure a variety of features, including survival, proliferation, and invasion [49,50]. However, it is likely that metabolic pathways involved in the expansion of the primary tumor are distinct from those that give advantage for metastatic progression. For instance, the tissue of origin of the primary tumor may influence the type of metabolic processes in action [51], given the variability in parameters such as organ vascularization and oxygen availability. Therefore, finding a pattern between cellular metabolism and metastatic intravasation in different cancer types is a challenge as well as an opportunity for future studies.

Glycolysis

One of the key players in cancer cell survival is glucose metabolism. Glucose serves as the main energy source in mammalian cells, by feeding into the glycolytic pathway. Indeed, cancer cells are commonly characterized by the Warburg effect [52]—their metabolism heavily relies on glycolysis, with the omission of the TCA cycle even in the high abundance of oxygen. However, how glucose metabolism affects metastasis, and intravasation specifically, needs to be addressed. Hexokinase 2 and pyruvate kinase 2 are the enzymes involved at the beginning and at the end of glycolysis, respectively. Their expression levels are correlated with motility and invasiveness as measured in *in vitro* and *in vivo* transplantation assays, and there is also a correlation between their levels in cancer tissue and disease aggressiveness in patients [53–59]. Furthermore, a byproduct of glycolysis, methylglyoxal, may be capable of inducing YAP-mediated metastasis, as shown *in vitro* and in *in vivo* transplantation assays [60]. Additionally, heavy reliance on glycolysis results in the accumulation of lactate. In a breast cancer mouse transplantation model, L-lactate did not affect tumor growth, but significantly increased metastatic potential

[61]. Lactate production may be involved in pH change that could stimulate activation of cathepsins and mmp9, which degrade ECM and promote invasion [62]. Indeed, inhibition of MCT1 lactate transporter could be a good therapeutic target as shown *in vitro* and *in vivo* transplantation model of breast cancer [63]. Interestingly, a recent study showed that pyruvate uptake induces the production of α -ketoglutarate, which remodels collagen via increasing the activity of the enzyme collagen prolyl-4-hydroxylase (P4HA). Inhibition of pyruvate metabolism was sufficient to impair collagen hydroxylation and decrease the metastatic burden of breast cancer in different mouse models [64].

Mitochondrial metabolism

Parallel with the reports of reliance of cancer cells on glycolysis, oxidative phosphorylation still plays a significant role, though the extent of this process may be variable in different cancer types [65]. Using isotope tracing, glucose oxidation was found to be low in clear renal cell carcinoma as studied in patients [66], while both glucose oxidation and glycolysis were high in lung cancer in patients, and glioblastoma models in mice [67–69]. Importantly, one of those studies revealed metabolic heterogeneity within tumor cells with respect to both glycolysis and glucose oxidation [68]. The question arises about the extent of cellular metabolic heterogeneity within human tumors, and more specifically, whether enhanced oxidation is of an advantage/disadvantage for metastatic intravasation.

In general, reports about the effect of mitochondria function on cancer cell invasiveness have been conflicting. Mitochondrial biogenesis and oxidative metabolism have been shown to promote metastasis in breast cancer *in vivo* models [70–72] with enhanced oxidative phosphorylation observed in breast cancer CTCs in patients [70], as well as to suppress it based on a study of prostate and melanoma models [73,74]. These studies relied on knockdown and overexpression of PGC1 α that mediates oxidative phosphorylation, and therefore, the discrepancy may result from tissue specific differences in endogenous levels of PGC1 α . It is possible that there is an optimal range of PGC1 α levels that promotes metastasis. It is also likely that cancer cells display a significant level of metabolic flexibility during disease progression and respond to other metabolic cues: For example, even though the Warburg effect is very common in cancer cells, alternative pathways could be activated when glycolysis is switched off. Indeed, oxidative phosphorylation was increased in cancer cell lines upon inhibition of glycolysis [75],

provision of low glucose concentration in the medium [76], and following induction of lactic acidosis [77]. Vice versa, suppression of PGC1 α -dependent mitochondrial oxidative metabolism brought up glycolysis in a melanoma cell line [78]. These findings emphasize the importance of single-cell resolution metabolic profiling of tumor cells along with matched circulating tumor cells to identify metabolic processes that may confer intravasation benefit.

Oxidative stress

Oxidative stress arises as a consequence of an imbalance between production and detoxification of reactive oxygen species (ROS), which are normally generated as by-products of oxygen metabolism. However, environmental stressors, which are prevalent during carcinogenesis, also contribute to the production of ROS [79]. ROS has been recognized as a metastasis promoting factor [71,80,81], and it could do so by promoting DNA damage or through the activation of prometastatic signaling pathways within cells [82,83]. For example, pharmacological scavenging of mitochondrial superoxide prevented metastatic spread from melanoma models in mice [71]. One study showed that mitochondrial DNA (mtDNA) of cells with high metastatic potential contained G13997A and 13885insC mutations in the gene encoding NADH (reduced form of nicotinamide adenine dinucleotide) dehydrogenase subunit 6 (ND6). These mutations resulted in a deficiency in respiratory complex I activity and consequently overproduction of ROS. When the endogenous mtDNA of poorly metastatic cells was replaced with that of a highly metastatic cell line of Lewis lung carcinoma, those recipient cells gave rise to more lung metastases as determined by intravenous (i.v.) injection assay [81]. Another study confirmed that the metastatic potential of melanoma cell lines, as revealed by i.v. assay, correlated with ROS levels [80]. However, both those studies examined the metastatic potential of cells without considering intravasation requirements, as the cells were injected directly into the circulation [80,81].

More recently, however, oxidative stress has been shown to inhibit metastasis and intravasation specifically, as blocking folate pathway and antioxidant treatment resulted in a higher number of CTCs and a higher incidence of metastases in melanoma models [84]. This antioxidative stress protection may happen via Nur77 and TGFB signaling as shown *in vitro* and in transplantation mouse models of melanoma [85]. Other studies have supported this notion of the negative effect of oxidative stress on intravasation in the

context of lung cancer [86–88] and melanoma [89]. It needs to be further defined if this factor could have its effect on metastasis via rendering the cells fit for intravasation and/or survival in the blood circulation. It may be that antioxidant metabolism facilitates cell survival by counteracting stress signals arising as a consequence of matrix detachment during intravasation. Altogether, while oxidative stress is generally considered to be metastasis-promoting, controversy is present and its specific role in the spontaneous intravasation of cancer cells *in vivo* remains to be fully dissected. The apparent disparity could, at least partially, result from different experimental approaches, using i.v. assays and tumor models to address this question [80,81,84–89].

Hypoxia and tumor vascularization

Hypoxia and vascularization, though apparently of opposite effect, are key features involved in cancer progression [11]. There is vast evidence for the role of hypoxia in metastasis promotion [90,91]. For example, induction of hypoxia in mouse tumor models using oxygen chambers resulted in increased metastasis to the lymph nodes in cervical carcinoma [92] and to distant organs in sarcoma [93]. Furthermore, other studies show a positive correlation between the extent of hypoxia in the primary tumor and metastatic rate as observed for mouse xenograft models of pancreatic cancer [94] and in patients with sarcoma [95]. High expression levels of hypoxia master regulators, Hif1 α and Hif2 α , correlate with a worse patient prognosis and a higher metastatic incidence in various cancers [90,96–99].

Focusing on intravasation, it has been shown *in vitro* that Hif1 α and Hif2 α reduce endothelial cell–cell adhesion via angiotensin-like 4 (ANGPTL-4) signaling and increase endothelial–cancer cell adhesion via induction of L1CAM on cancer cell surface [100], thus providing a possible mechanism of hypoxia-driven intravasation. Another study suggested that hypoxia could drive intravasation via signaling through CXCR4, which results in adhesion of cancer cells to endothelial cells and trans-endothelial migration in *in vitro* assays [101]. Hypoxia also induces secretion of VEGF, rendering blood vessels more permeable [90]. A study using a fibrosarcoma cell line found increased hypoxia, enhanced blood vessel permeability, and elevated intravasation rate at the core in a chick embryo mesodermal model of tumor growth [102]. These findings should also be verified with more standard approaches of *in vivo* orthotopic transplantation models. A recent study on breast tumor xenograft models

suggested that both central/core as well as scattered hypoxia areas are present in a model-dependent fashion. More importantly, however, the study provided a direct link between intravasation and hypoxia, by showing that CTC clusters arise from hypoxic areas. Interestingly, the authors show that *in vivo*, hypoxia results in cell–cell junction upregulation and intravasation of clustered CTCs with an elevated metastatic ability [103].

Fatty acid uptake

Interestingly, fatty acid metabolism has also been shown to have an effect on metastasis. A population of cells expressing high levels of CD36, a fatty acid translocase associated with stem cell-like features of some cancer cells [104,105], showed high metastatic potential, while stimulation of cells with palmitic acid increased their metastatic ability in orthotopic mouse models of human oral cancer, in a CD36-dependent manner [106]. Furthermore, an analysis of published patient datasets suggested that mutations in the pathways responsible for the uptake of free fatty acids correlated with invasiveness [107]. A possible mechanism of fatty acid action could be via their incorporation into membrane structure to induce oncogenic signaling [108], or via their oxidation and subsequent signaling [109]. These reports are very interesting and may suggest a more prominent role of fatty acid metabolism in the metastatic process than previously anticipated.

Outlook

While cancer cell metabolism has been extensively studied, highlighting intriguing roles for glycolysis, mitochondrial metabolism, antioxidant metabolism, tumor hypoxia, and fatty acid uptake, it is becoming increasingly clear that cancer cells are also heterogeneous in the context of their metabolic status, raising the intriguing question of whether or not altered metabolism can be intravasation-promoting in individual tumor ecosystems. Clearly, an answer to this question will require metabolic profiling of matched primary tumor cells, CTCs and metastatic deposits, possibly at the single-cell level. We speculate that such analyses should also be extended to cells that are part of the tumor microenvironment, as their metabolic activity could influence intravasation dynamics.

Cells in the microenvironment

Tumor microenvironment consists of a variety of cells including immune cells, endothelial cells, activated

fibroblasts, and pericytes. Here, we outline their potential involvement in the intravasation process. Their mechanisms of action include modification of cancer cell properties via signaling pathways, altering the tumor microenvironment or direct participation in the intravasation process. While the effects of individual groups of cells are discussed below, future research efforts should also focus on their cooperative activity.

Cancer-associated Fibroblasts

Fibroblasts are residents of many tissues, and during cancer development they can become activated or recruited to the tumor site, and as such they are referred to as cancer associated fibroblasts (CAFs) [110]. This population of cells has been associated with various aspects of tumor progression including tumor expansion, extracellular matrix (ECM) remodeling, angiogenesis, invasiveness, and metastasis [111]. Both progression-promoting and inhibitory roles in metastasis have been reported [112,113]. It is therefore crucial to identify subpopulations of CAFs and their respective functions.

CAFs were observed to be part of CTC clusters in efferent blood from tumors, and partial depletion of CAFs in mice resulted in reduced metastatic burden to the lungs [114]. Additionally, an observation was made that lung carcinoma as well as brain metastases in patients contained CAF populations within their microenvironment, while no fibroblasts were detected in healthy brain tissue or in primary brain cancers [114]. In line with the above, CAFs have also been detected as part of CTC clusters in the peripheral blood circulation in patients [115] and may confer shear resistance to CTCs as studied in *in vitro* systems [115]. Altogether, these studies suggest that CAFs may be associated with CTCs and potentially involved in their intravasation. Further evidence for CAF involvement in this process comes from the analysis of primary tumors. Fibroblasts were observed at the leading edge of invasive cancers, surrounding cancer cells in ‘collective invasion packs’ *in vivo* [116]. The cells at the leading edge have been shown to invade together by means of heterotypic N-cad/E-cad-based adhesion between CAFs and cancer cells in 3D/2D migration assays and supported by protein expression analysis in patient tumor samples [117]. Altogether, these results suggest a potential role for CAFs in the intravasation process, as well as, more broadly, in metastatic cancer progression, and their full characterization in this regard is likely to reveal important mechanisms of cancer spread.

Neutrophils

Neutrophils are cells of the innate immune system that abundantly infiltrate the developing tumor. For long, they have been considered as bystanders; however, the past decade has provided evidence that in fact they play a very important role in tumor development and progression [118–120]. Neutrophils may indeed promote metastasis, especially by creating a premetastatic niche in the distant organs [121,122]. One way in which the neutrophils could exert their prometastatic function is via release of neutrophil extracellular traps (NETs), which are commonly produced to trap microorganisms and consist of chromatin DNA filaments coated with granule proteins. Indeed, recently, NETs have been detected in metastatic lesions and to a lesser extent in primary tumors of breast cancer patients [123]. NETs have been shown to be implicated in the formation of a premetastatic niche in the liver and their removal impaired metastasis formation *in vivo* [123], while the role of NETs at other stages of the metastatic cascade and, particularly during intravasation, needs to be investigated. More generally, the presence of neutrophils has been previously correlated with increased intravasation rates *in vivo* [124], but recently it has been directly shown that these cells participate in the intravasation of CTC clusters in breast cancer patients and mouse models, particularly by partnering with cancer cells and sustaining their cell cycle progression in circulation, leading to increase metastatic propensity [7].

Macrophages

Macrophages are the cells of the innate immune system, and when recruited to the cancer site, they are referred to as tumor associated macrophages (TAMs). Macrophages have been shown to display both pro- and antitumorigenic functions [125].

Direct insight into the role of macrophages in intravasation was given by the discovery of the Tumor Microenvironment of Metastasis (TMEM) Doorway, composed of a Tie2-high VEGFA-high perivascular macrophage, a tumor cell expressing high levels of the actin-regulatory protein mammalian enabled (Mena), and an endothelial cell as functional ‘doorway’ for hematogenous dissemination. Tie2-high VEGFA-high perivascular macrophages cause transient vascular permeability and result in intravasation of tumor cells as shown in the MMTV-PyMT breast cancer model and in the PDX TN1 xenograft model [126–128]. Moreover, macrophages enhance the

performance of cancer cells in *in vitro* transmigration assays [4] and promote invadopodia formation in a Rho-A signaling-dependent fashion [4] or via Notch1/Mena signaling pathway [129]. Macrophages have also been implicated in promoting early dissemination of cancer cells in breast cancer model [130]. In contrast, some other studies have shown a tumor-inhibitory function for macrophages. This dual nature of macrophages (pro- vs antitumor) in cancer progression may arise from the existence of different functional subtypes, including the M1 and M2 macrophages that are described to be of proinflammatory and immunosuppressive function, respectively [131,132]. Altogether, there is strong evidence that macrophages may play a significant role in the intravasation of cancer cells. The involvement of specific subpopulations of macrophages needs to be determined for designing efficient antimetastatic therapies that act at the macrophage level. Furthermore, it will be essential to understand fully the involvement of macrophages in concert with other tumor-associated cell types [131], while considering macrophage heterogeneity and the possibility that different macrophage subtypes may intervene at different stages of the metastatic progression.

T cells

T lymphocytes are cells of the adaptive immune cells that commonly infiltrate the tumor environment during disease progression [133,134]. While cytotoxic CD8⁺ T cells are believed to exhibit antitumor activity, helper CD4⁺ T cells have been suggested to promote cancer progression in some contexts. The link between these cell types and intravasation is beginning to be resolved.

CD4⁺ T lymphocytes have appeared as cells contributing to enhanced intravasation via direct signaling to cancer cells [135] or via indirect effect through regulation of the TAMs [136]. These studies have used mouse models of mammary cancer combined with depletion and inactivation of lymphocyte subpopulations, followed by analysis of metastasis to the lungs and count of CTCs. However, a more recent study has suggested that CD4⁺ T lymphocytes may be involved in vasculature normalization, and therefore, via this mechanism they could impede cancer cell intravasation [137]. The role of CD8⁺ T cells in intravasation has been investigated to a lesser extent; however, a recent study has demonstrated that tumors in CD8 knockout mice and mice with CD8 inactivation give rise to more metastases and increased numbers of CTCs [138]. This suggests that

CD8⁺ cells play a safeguard role to intravasation. To corroborate these findings, it would be essential to understand the role of various subpopulations of CD4⁺ and CD8⁺ T cells in different contexts and stages of metastatic progression.

Platelets

Platelets have been implicated in angiogenesis and metastasis promotion [139,140]. One study suggested that platelets induce EMT in cancer cells *in vitro* and improve extravasation by means of i.v. injection in mice [141]. While these studies highlight the involvement of platelets in the later stages of the metastatic cascade, it is possible that platelets also play an important role in the early events. For example, their involvement at the intravascular transit should be considered, both from the blood stream as well as from the tumor site [140]. Notably, platelet signatures have been consistently found in both mouse and human CTC clusters [6,7].

Pericytes

Pericytes are commonly associated with endothelial cells, and are considered to be vital regulators of angiogenesis and vascular stability in both healthy and pathological conditions. Recruitment of pericytes to newly formed vessels in tumors has been shown to impede their sprouting, enhance vessel maturation, and decrease their leakiness [142,143]. As such, they may be considered as gatekeepers of metastasis.

This notion is supported by the analysis of patient samples, which showed that low pericyte coverage of blood vessels is associated with a drop in survival in invasive breast cancer patients [144–146], and it is further corroborated by various mouse model studies. For instance, mice deficient in endosialin, a pericyte and myofibroblast marker [147–149], showed an increased metastatic rate with no effect on tumor growth and no effect on extravasation as studied by i.v. injection in mice [150]. Additionally, enhanced metastasis accompanied by overall tumor growth reduction in mice was observed following depletion of Ng2⁺ pericytes [151], and in PDGFR- β ret/ret mouse model (bearing a truncating mutation in PDGFR- β , a putative pericyte marker) [152]. Conditional inactivation of Shb—an adapter protein downstream of tyrosine kinases such as VEGFR2 and PDGFR—in pericytes with the PDGFR- β -CreERT2 reporter system resulted in a decreased pericyte coverage of small tumor vessels, increased leakage, and higher metastatic rate without affecting tumor growth [153]. This

reinforces the notion that pericytes may be involved in intravasation by influencing blood vessel wall leakage. It should be noted, however, that part of the effect observed with the PDGFR- β -CreERT2 reporter system may be due to the fact that PDGFR- β promoter may also be active in other cell types such as CAFs [154–156] or tumor cells themselves [157,158]. Another study supports the hypothesis that pericytes influence endothelial wall leakiness, which was observed in response to the deletion of the A6-integrin subunit of A β 1 integrin. Surprisingly though, while reduced pericyte coverage of tumor vessels and enhanced leakiness were observed, other parameters such as tumor growth, blood vessel density, and metastases were not affected [159].

On the opposing side, a study has suggested that orthotopic colon cancer grown in endosialin-negative context resulted in a reduced tumor growth, invasion, and metastatic burden compared with wild-type mice [160]. Another article showed that tumor cells may influence pericytes to convert into fibroblast-like cells, thereby enhancing the invasiveness and intravasation of cancer cells [161]. Altogether, it is possible that the role of pericytes, and ultimately their subpopulations, may be context-dependent. Indeed, the extent of pericyte coverage, and consequently their collective effect, could be variable with respect to tumor type and its location [162], as well as tumor progression stage [163]. Furthermore, the functional heterogeneity of pericytes based on different marker expression [143] should be carefully examined in future studies, as this could potentially reveal differential effects of pericyte subpopulations.

Adipocytes

Adipocytes are the main constituents of adipose tissue, and their function is to store fat as well as to secrete lipid and protein factors [164]. They have drawn attention in recent times in the context of growing obesity rates, and the observed correlation between obesity and cancer incidence [165]. Additionally, they are the major cellular component of breast tissue and get activated during cancer tumorigenesis, that is, become cancer associated adipocytes [166]. They are also a prevailing cell type in melanoma [167]. Cancer-associated adipocytes have been implicated in cancer development and metastasis [166,168,169]. *In vitro* studies suggest that adipocytes may indeed promote cancer cell migration and invasion, and several mechanisms have been proposed. Coculture of ovarian cancer cells with adipocytes increases the proliferation of cells *in vitro* and the

growth of those cells in transplantation assays *in vivo* and may promote their metastasis by inducing *Feb4* expression in cancer cells [169,170]. Similarly, the proliferation, invasion, and migration of melanoma and colon cancer cell lines *in vitro* were increased due to adipocyte influence [171]. Adipocytes may also act on cancer cells via the transfer of vesicles that contain enzymes involved in fatty acid oxidation [172], or via lipid transfer from adipocytes to melanoma cell lines *in vitro* and in Zebrafish transplantation model [173]. Finally, adipocytes may act to remodel collagen and in this way enhance migration and intravasation of cells [168]. More *in vivo* mouse models and analyses of patient samples will be necessary to assess whether and how adipocytes directly affect intravasation.

Outlook

In recent years, significant progress has been made in the understanding of the effect that different cell types within the tumor microenvironment have on metastatic progression, including intravasation. It will be interesting to gain a high-resolution insight into the involvement of various microenvironmental cell types in different steps of intravasation—their contribution to the initial migration or detachment of cancer cells, cancer cell interaction with the endothelial wall, and during endothelial transit. Interactions between different cell types and their collective effect on intravasation should also be considered.

Mechanical cues

Cancer is confined by the tissue in which it resides ‘with limited inlets and outlets for cells, fluids and waste’ [174]. When cancer invades the surrounding tissue, it causes a buildup of pressure. Biomechanical abnormalities in tumors consist in elevated solid stress, elevated interstitial fluid pressure, increased stiffness, altered material properties, and altered tissue microarchitecture [175]. These mechanical cues have an effect on other cancer-associated features, for instance, influencing microenvironment components, inducing inflammation, and promoting abnormal signaling.

There is evidence that mechanical cues may affect metastasis, but how precisely, and by which mechanical, cellular and molecular mechanisms, remains to be discovered. YAP and TAZ, transcriptional coactivators that respond to various stimuli including the hippo pathway or mechanical signals, are commonly hyperactivated in human cancers. However, no activating mutations have been detected, and hippo pathway

mutations are rare [176]. Therefore, it is possible that YAP/TAZ activation is induced by mechano-stimuli within the tumor. For example, cytoskeleton arrangements could be involved in the activation of YAP/TAZ [176]. Because of their role in mechano-transduction [176,177], YAP-TAZ involvement in different steps of the metastatic cascade—including intravasation—should be studied mechanistically and functionally.

Aggregation of ECM proteins may result in stiffening, which is correlated with poor patient prognosis [178]. ECM stiffening may be due to activity of cancer cells and cells of the microenvironment such as macrophages and CAFs [179]. For example, stiff ECM may induce CAF activation in a YAP/TAZ-dependent manner, which then further contributes to ECM stiffening in a positive feedback loop [180]. Reports have shown that stiffness of matrices is linked to cancer cell invasiveness [181–183] and their metastatic potential *in vivo* [184], though some other studies suggest that the migration velocity of cells of higher metastatic potential is independent of matrix stiffness [185] or may rather depend on the matrix molecular composition [186]. How exactly stiffness can affect cancer cell intravasation remains to be clarified.

Interstitial fluid pressure (IFP) buildup is another occurrence in cancer. In healthy tissue, influx of plasma from the blood vasculature with nutrients and oxygen is used for metabolism of cells and is then reabsorbed by post-capillary venules, while a fraction drains through the lymphatic system to reach the blood stream. In cancer, the amount of fluid is very high and not drained well due to various abnormalities in the vasculature, lymphatic co-option, and increase in cell number and density [174]. IFP may have an effect on metastasis: for example, reducing interstitial fluid pressure with gelatin modified cationic lipid particles leads to decreased pulmonary metastasis in an orthotopic breast cancer model [187]. Xenografts of cervical and melanoma cell lines and patient tumors show a correlation between IFP level and metastasis [188,189]. It would be crucial to understand how these mechanical signals translate into molecular changes within cancer and stromal cells and feed into the enhancement of the intravasation process. Contradictory with the mentioned observations, another study suggests a lack of correlation between IFP of tumor or lymph node metastasis and lungs in mouse models [190]; however, the dissemination of cells from minority high-IFP areas, that are not evident in the average IFP measurement, could take place.

Outlook

The effect of mechano-stimuli on cancer progression is a developing field and the link between mechanical stimuli and intravasation should be rigorously investigated, possibly using spontaneous metastasis models *in vivo* as well as dedicated reporter systems to measure the activity of mechano-sensing pathways during cancer progression.

Concluding remarks

Several processes and factors have been highlighted in the context of metastatic intravasation (Fig. 3). Collectively, intravasation is mostly referred to and generally associated with the process in which cancer cells enter the bloodstream from a primary tumor site. However, it is important to highlight that intravasation could also occur from established metastatic deposits and be the key event in metastasis-to-metastasis dissemination of cancer. Clearly, intravasation biology could be organ-dependent, with different mechanisms being involved at distant sites as a consequence of varying tissue properties. Generally, in recent years, various studies have suggested a role for immune cells or genetic and gene expression profiles in the intravasation process. At the same time, other areas, such as epigenetic, metabolic, and mechanical inputs, are being investigated. Focusing future research efforts on elucidating the drivers of the intravasation process, individually and in combination, in an organ-specific fashion and using spontaneous metastasis models *in vivo* and patient samples will be instrumental in dissecting the dynamics of metastasis and more importantly, in

identifying new treatment opportunities for patients with aggressive cancers.

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Conflict of interest

N. Aceto is listed as inventor in patent applications related to CTCs and is a paid consultant for companies with an interest in liquid biopsy. M.K. Sznurkowska declares no competing financial interests.

Author contributions

M.K. Sznurkowska and N. Aceto wrote the manuscript and created the figures.

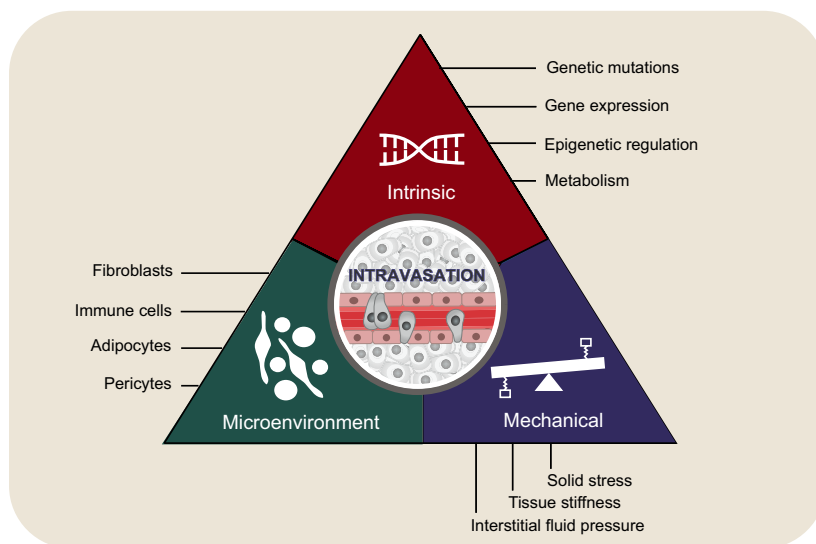


Fig. 3. Summary of factors involved in the regulation of intravasation process: intrinsic cancer cell properties, cellular microenvironment, and mechanical properties.

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