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Cancer cells remodel themselves and vasculature to overcome the endothelial barrier

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Abstract

Metastasis refers to the spread of cancer cells from a primary tumor to distant organs mostly via the bloodstream. During the metastatic process, cancer cells invade blood vessels to enter circulation, and later exit the vasculature at a distant site. Endothelial cells that line blood vessels normally serve as a barrier to the movement of cells into or out of the blood. It is thus critical to understand how metastatic cancer cells overcome the endothelial barrier. Epithelial cancer cells acquire increased motility and invasiveness through epithelial-to-mesenchymal transition (EMT), which enables them to move toward vasculature. Cancer cells also express a variety of adhesion molecules that allow them to attach to vascular endothelium. Finally, cancer cells secrete or induce growth factors and cytokines to actively prompt vascular hyperpermeability that compromises endothelial barrier function and facilitates transmigration of cancer cells through the vascular wall. Elucidation of the mechanisms underlying metastatic dissemination may help develop new anti-metastasis therapeutics.

Keywords

metastasis; EMT; vasculature; intravasation; extravasation; transendothelial migration

1. Introduction

Metastasis is the major reason for cancer-related deaths. It is a sequential order of events that involves invasion and migration of cancer cells from the primary site to distant organs where they eventually form secondary tumors. This multi-step process is referred to as invasion-metastasis cascade [1–3] and could be simplified into two phases: 1. *Physical translocation* of a cancer cell from the primary tumor to the distant tissues (secondary site). 2. *Colonization* of these cancer cells at the secondary site [4]. The first phase generally encompasses: a. *Epithelial-to-mesenchymal transition (EMT)*, b. *Intravasation* (entry of

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Conflict of interests

The authors declare there is no conflict of interest.

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cancer cells into the blood vessels), and c. *Extravasation* (exit of cancer cells from the blood vessels to invade secondary organs). EMT is a process during which epithelial cells lose their cell-cell adhesion and polarity to transform into mesenchyme-like cells [5,6]. The hallmarks of EMT include loss of cell-cell junctions, loss of apical-basal polarity, and acquisition of migratory and invasive property [7]. Therefore, cancer cells that have undergone EMT (EMT-cancer cells) are more motile and inclined to invade the surrounding tissues. EMT-cancer cells accomplish this either as single cells or collectively in small groups [8,9]. Following local invasion, EMT-cancer cells intravasate, resulting in dissemination of cancer cells into the blood stream [9]. Once in blood circulation, these cancer cells are referred to as circulating tumor cells (CTC). CTCs hold a prognostic significance in cancer progression as they determine the ability of the primary tumors to form distant metastatic lesions [10,11]. CTCs that survive the shear stress and immune reactions in the blood stream extravasate through the blood vessels to colonize metastatic sites [12]. An important feature that is common to the intravasation and extravasation processes is transendothelial migration (TEM). TEM is a phenomenon by which the EMT-cancer cells cross the vascular endothelial layer [13]. Endothelial cells (ECs) normally serve as a barrier to the movement of cells into or out of the blood. However, metastatic cancer cells do cross the vascular endothelial layer during intravasation and extravasation, suggesting that the integrity of vasculature at the primary and metastatic sites may be impaired in order to facilitate TEM. In this review, we will summarize current understanding on how the process of EMT in cancer cells and the favorable modulation of vascular properties by tumor cells play a critical role in cancer cell TEM, dissemination, and metastasis (Fig. 1).

2. Epithelial-to-mesenchymal transition (EMT)

Epithelial cells are structurally well-defined cells that are linked to each other through multiple types of intercellular junctions, including adherens junctions, desmosomes, and tight junctions, to form single cell layer or multilayered tissues. These cells maintain apical-basal polarity by anchoring themselves to the underlying basement membrane via hemidesmosomes [14]. Thus, epithelial cells function as permeability barriers and play a crucial role in tissue organization and organ delineation [15]. In contrast, mesenchymal cells seldom establish tight contact with neighboring cells and are often found embedded inside the extracellular matrix (ECM). Epithelial cells exhibit remarkable plasticity and are able to transform into spindle-shaped mesenchymal cells through the process of EMT. Depending on tissue and signaling milieu, EMT-cancer cells may completely or partially lose epithelial characteristics [16]. During EMT, epithelial cells lose junctional contacts and reorganize their cytoskeletal architecture. They also gain front-rear polarity, and become highly motile and invasive. At the molecular level, EMT is characterized by downregulation of epithelial genes such as E-cadherin, desmoplakin, and epithelial cytokeratins and activation of mesenchymal genes like N-cadherin, vimentin, fibronectin, and smooth muscle α actin (α -SMA) [17,18]. The repression of epithelial markers and activation of mesenchymal genes are controlled by several transcription factors such as SNAIL and SNAIL2/SLUG, ZEB1 and ZEB2/SIP1, and TWIST1 that are the master regulators of EMT [17–19]. Various signaling

molecules and pathways induce EMT in cancer cells by finely regulating these transcriptional factors and promote cell migration and invasion [16].

EMT facilitates cancer cell migration within the tissues either individually or collectively by forming chains of migratory cells. Due to the migratory nature of mesenchymal-like EMT cells and their flexibility to revert back into epithelial cells by mesenchymal-to-epithelial transition (MET), EMT constitutes an integral part of development and wound healing [7,16]. Furthermore, molecular mechanisms dictating EMT are reactivated in pathological processes like fibrosis and cancer where it contributes significantly to disease pathogenesis [20,21].

The vast majority of solid tumors are epithelial in origin [22]. Depending on the microenvironment and signaling context, these cells readily undergo EMT. EMT is critical for cancer cells to display metastatic properties like invasion, intravasation and extravasation, all critical in the first phase of metastasis. Invasion involves infringement of cancer cells from the confined region into neighboring tissue parenchyma [3]. Cancer cells achieve this by forming invadopodia [23]. Invadopodia is a special phenotypic and functional characteristic acquired by cancer cell upon EMT. Invadopodia facilitates breaching through the basement membrane (BM), which is made up of specialized ECM comprising of a mixture of tethering molecules [23]. To digest the ECM and invade the surrounding tissue, cancer cells make use of various matrix metallo-proteinases (MMPs) [24]. These MMPs in cancer cells are upregulated as a consequence of EMT [18,25]. Moreover, several studies have demonstrated that EMT confers tumor-initiating property on cancer cells, which are referred to as cancer-stem cells (CSC) [26–29]. These CSCs form a small subset of tumor cells similar to the small percentage of cells that invade and metastasize [30]. Furthermore, accumulating evidence indicates an unequivocal role of CSCs in invasion [31,32]. The fact that EMT imparts cancer cells with stem cell properties and CSCs are important in metastasis suggests an important role of EMT in invasion and metastasis. *In vitro* and *in vivo* experimental models provide additional evidence for EMT being critical for invasion and dissemination of cancer cells. Depletion of EMT-cancer cells in transgenic breast cancer mouse model reduces metastatic lesions [33]. In addition, TWIST1-induced EMT in cancer cells results in cancer cell dissemination in spontaneous squamous cell carcinoma mouse model. Thus, the process of EMT is a prerequisite for cancer cell invasion, migration and metastasis. However, for the second phase of metastasis, reversal of EMT (i.e. MET) is required for the tumor cells in circulation to colonize and form metastatic lesions [34]. Here, we would focus on discussion of the first phase of metastasis, in particular EMT and vascular modulation. Readers can benefit from detailed discussion of MET and colonization in several excellent reviews [14,35,36].

Occurrence of EMT in cancer cells is highly dependent upon the signaling molecules that are secreted by tumor microenvironment or by cancer cells themselves. We will discuss several important signaling molecules and pathways that induce EMT to promote cell migration and invasion. In addition, we will touch upon different proteases secreted by tumor cells that augment the invasive ability of the EMT-cancer cells.

2.1 Signaling molecules and pathways involved in EMT

Signaling pathways that induce EMT also confer migratory and invasive capacity on cancer cells. Additional factors like proteases may further enhance these properties. As intravasation requires cancer cells to invade through the tissues to reach the blood vessels, it could be difficult to pinpoint which signaling molecules or pathways are specifically involved in intravasation rather than invasion. Indeed, the signaling molecules and pathways involved in EMT, migration, invasion and intravasation overlap extensively. These pathways also crosstalk to a great extent, making it difficult to delineate the individual role of each pathway in the metastatic process. Considering these limitations, we select pathways in the context of EMT and its associated effects during metastasis.

2.1.1 TGF β —TGF β has long been regarded as a crucial factor in the process of EMT in both development and cancer [5,37,38]. Out of the 3 types of TGF β s (TGF β 1, 2 and 3), TGF β 1 has been linked to EMT induction during cancer progression [16]. TGF β 1 has been shown to induce transdifferentiation of mammary epithelial cells into mesenchymal cells *in vitro*. This transformation was associated with a decrease in the expression of epithelial markers and an increase in the expression of mesenchymal markers [39]. TGF β 1-transgenic mice exhibit a malignant phenotype of skin cancer with increased incidence of spindle cell carcinomas [40]. Several studies have implicated TGF β in various cancers like hepatocellular, breast, prostate, lung and colon cancers for conferring a malignant and invasive phenotype on tumor cells [41–45]. TGF β is secreted by both cancer and stromal cells [46]. Secreted TGF β mediates its function in an autocrine or paracrine fashion. For instance, TGF β -induced EMT in RAS-transformed mouse mammary epithelial cells maintains the mesenchymal state by activating the autocrine signaling loop of TGF β [47]. In another study, spontaneously transformed mesenchymal subpopulation of immortalized human mammary epithelial (HMLE) cells and TWIST-overexpressed HMLE cells secrete high levels of TGF β when compared to the parental HMLE cells, and the TGF β autocrine signaling helps maintain their mesenchymal phenotype [48]. TGF β secreted by cancer cells also exhibits paracrine signaling by inducing myofibroblastic phenotype in quiescent fibroblasts [49–51] that would produce and modify the ECM and promote invasion [52]. However, primary culture data with human kidney tubular epithelial cells support the role of TGF β only in EMT but not in myofibroblast transdifferentiation [53].

TGF β generally acts through a receptor complex that activate the downstream component SMADs [54]. Activated SMADs in turn induce the expression of EMT transcription factors such as SNAI1 [55], SLUG [56], ZEB1 [57] and TWIST [58] that further amplify the EMT-associated molecular changes and facilitate cancer cell invasion and dissemination. Altering the expression of various SMADs prevents TGF β -induced EMT [16]. Apart from SMADs, TGF β also relays its effects through RHO-like GTPases, Erk, p38, PI3K and MAPK signaling pathways to facilitate EMT and its associated events [59]. Moreover, TGF β is known to activate or act in concert with other EMT-inducing signaling pathways, including Wnt, Notch, and integrin signaling, to trigger EMT [60].

2.1.2 HIF1 α —Hypoxia-inducible factor 1 α (HIF1 α) protein is highly unstable under physiological conditions, but becomes stabilized upon hypoxia [61]. The majority of the

hypoxia- dependent effects in cancer are mediated by HIF1 α [62]. A study by Cooke et al. shows that hypoxic tumor environment spawned by pericyte depletion stimulates HIF1 α protein accumulation, which in turn induces EMT to promote cancer metastasis [63]. Conversely, conditional depletion of HIF1 α in the mammary epithelium of a transgenic mouse model for metastatic breast cancer decreases pulmonary metastasis [64]. Similarly, inhibition of HIF1 α in pancreatic cancer cells impairs their migration and invasion, leading to reduced metastasis [65]. HIF1 α expression also shows a correlation with poor prognosis and metastasis in cancer patients [66–68]. All these studies indicate an indispensable role of HIF1 α in hypoxia-induced EMT and metastasis. These effects of HIF1 α are in a large part attributed to its target genes, such as TWIST and SNAIL. HIF1 α is shown to regulate the expression of TWIST by binding directly to the hypoxia-response element in the TWIST promoter. Depletion of TWIST in HIF1 α -overexpressing or hypoxic cells reverses the EMT and metastatic phenotype, indicating that TWIST is an essential effector molecule of HIF1 α -mediated metastasis [69]. Hypoxia-induced EMT in hepatocellular carcinoma has been demonstrated to be mediated by HIF1 α via the activation of SNAIL [70].

2.1.3 Wnt—Both β -catenin-dependent (canonical) and -independent Wnt signaling pathways have been implicated in EMT, invasion, migration and metastasis. Activation of the Wnt/ β -catenin pathway has been shown to promote the transcription of several EMT-inducing transcription factors [71]. However, activation of the Wnt/ β -catenin pathway alone may not be sufficient to induce EMT [60]. Nevertheless, Wnt/ β -catenin signaling can act in concert with other oncogenic pathways to orchestrate the EMT program. For example, activated Wnt/ β -catenin signaling along with activated KRAS oncogene in lung epithelium produces a more aggressive tumor phenotype by decreasing E-cadherin expression [72]. Similarly, β -catenin signaling promotes lung metastasis in the NRAS-driven murine model of melanoma [73]. Activation of Wnt/ β -catenin signaling has been implicated in bone metastasis during multiple myeloma, prostate and breast cancer progression [74]. Moreover, activated Wnt/ β -catenin pathway is identified as a determinant of metastasis in lung adenocarcinoma, because inhibition of the pathway attenuates the ability of lung cancer cells to form brain and bone metastases in mice [75]. Consistent with these observations, a functional blockade of Wnt/ β -catenin pathway by either a pharmacological Wnt-antagonist, or siRNA mediated inhibition of β -catenin decreases metastasis-associated phenotypes including fibronectin-directed migration, F-actin organization, and invasion in triple negative breast cancer (TNBC) cells [76].

In comparison with the Wnt/ β -catenin pathway, the importance of β -catenin-independent Wnt signaling in metastasis is poorly investigated. However, Wnt signaling through ROR1/2 receptor has been demonstrated to play a critical role in brain metastasis during basal-like breast cancer progression [77]. Similarly, knockdown of ROR2 in melanoma cells correlates with reduced frequency of lung metastasis [78]. Wnt ligands along with frizzled receptor have also been implicated in cell migration and invasion in a β -catenin-independent manner (reviewed by Anastas et al [79]).

2.1.4 Notch—Notch signaling plays an important role in EMT regulation during normal development and cancer [80,81]. Notch proteins are transmembrane receptors that interact

with transmembrane ligands Delta like (DLL) or Jagged. Notch and DLL/Jagged interaction activates the pathway by releasing the intracellular domain of Notch, which subsequently translocates into the nucleus to bind to CBF-1-Suppressor of Hairless/Lag1 (CSL), and recruits coactivators such as Mastermind-like (MAML) proteins. The so formed Notch–CSL–MAML complex in the nucleus activates the downstream targets including Hes, Hey, cyclinD1 and c-myc [82]. Aberrant Notch signaling due to increased expression of either Notch receptors or Jagged ligands or Notch target genes has been implicated in inducing EMT and promoting metastasis in various cancers including osteosarcoma, melanoma, breast, prostate, pancreatic, gastric and colorectal cancer [83]. Furthermore, Notch signaling has been linked to therapeutic resistance with increased incidence of EMT. For instance, Notch4 together with Nicastrin promotes endocrine therapy resistance and EMT in MCF7 breast cancer cells [84]. Thus, targeting Notch signaling may not only sensitize the tumors to therapies but also reduce the occurrence of EMT.

Apart from the aforementioned prominent pathways, several other signaling pathways such as Hedgehog, RAS, SRC, PI3K/AKT, JAK/STAT, and Bone morphogenetic protein (BMP), have also been implicated in the induction of EMT and promotion of metastasis (Summarized in Table 1) [9][16]. These pathways function by decreasing the expression of E-cadherin either directly or indirectly by regulating the expression of EMT transcription factors SNAI1, SLUG and ZEB [85].

2.2 Proteases that induce EMT and facilitate cell invasion

Several types of proteases like MMPs and cathepsins have been implicated in EMT and cell invasiveness. MMPs are a family of zinc-dependent endopeptidases involved in ECM remodeling, and degrade almost all components of ECM such as gelatin, collagen, fibronectin, tenascin etc. MMPs are involved in various physiological and pathological processes including invasion and migration of cancer cells in malignant carcinomas [99,100]. MMPs have been shown to induce EMT in several types of cancer. MMP3 induces EMT by activating SNAI1 expression in a mouse mammary epithelial cell line [101]. MMP9 together with SNAI1 has been shown to induce EMT in epidermoid carcinoma cell line [102]. Apart from EMT, MMPs secreted by cancer cells are involved in the induction of invasive and migratory properties. A recent publication shows that membrane palmitoylated protein 3 (MPP3), a member of MAGUK family, increases the invasiveness and migration of hepatocellular carcinoma cells by upregulating MMP1 [103]. RANKL/RANK in breast and prostate cancer cells have been shown to upregulate MMP1 to promote invasiveness *in vitro* [104]. Furthermore, the tetraspanin CD81 protein in melanoma increases cell motility by up-regulating metalloproteinase MT1-MMP expression through the pro-oncogenic AKT-dependent signaling [105]. Moreover, elevated levels of MMP2 and MMP9 have been associated with low differentiation grade and accelerated tumor progression in oral, lung, bladder, ovarian, papillary and thyroid carcinomas [99].

Cathepsins are a group of lysosomal proteinases or endopeptidases that belong to a diverse number of enzyme subtypes, including cysteine proteases, serine proteases and aspartic proteases [106]. Cathepsins are secreted by cancer cells as well as the stroma, and are implicated in cancer progression and invasion [107,108]. In addition to degrading the ECM

on their own, Cathepsins can initiate a proteolytic cascade by activating other proteases such as MMPs and urokinase plasminogen activator, which in turn promote invasion [109]. Therefore, these proteases not only promote invasion by inducing EMT in cancer cells but also promote invasion and migration by degrading and remodeling the ECM.

3. Tumor vasculature and its role in metastasis

Tumor vasculature is part of tumor microenvironment. Vascular ECs have also been involved in modulation of cancer cells to consequently enhance their invasive and migratory ability. ECs have been shown to induce EMT in human pancreatic, lung and mouse breast cancer cell lines by constitutive secretion of TGF β 1 and TGF β 2 [110]. Sigurdsson et al. shows that ECs are potent inducers of EMT in basal-like breast cancer cells [111]. ECs also induce EMT in squamous cell carcinoma (SCC) cells by secreting epidermal growth factor (EGF). The authors showed that SCC cells within the perivascular region exhibit the EMT phenotype in primary SCC tissue from patients who developed distant metastases [112]. EC-secreted fibronectin extra domain A (EDA) promotes the metastatic capacity of colorectal cancer cells (CRC) by inducing EMT via the interaction with integrin α 9 β 1 on CRC cells. This interaction activates FAK and Rac signaling, resulting in polarized cytoskeleton that promotes the invasive capacity of CRC cells [113].

Like epithelial cells, ECs can transition into mesenchymal-like cells, and this phenomenon is referred to as endothelial to mesenchymal transition (EnMT). During EnMT, ECs lose endothelial junctions, acquire mesenchymal markers, and develop invasive and migratory abilities [16,114]. EnMT abolishes vascular integrity and facilitates cancer cell migration across the vascular endothelial layer. TGF β and Notch signaling pathways play a critical role in EnMT [115]. EnMT is a well-known source that contributes to the formation of cancer-associated fibroblasts (CAFs) [114]. These CAFs not only provide necessary signals for cancer cell dedifferentiation, proliferation, and survival, but also facilitate metastasis by modulating the tumor immune microenvironment [116,117]. Therefore, ECs indirectly contribute to invasive and migratory properties of cancer cells by undergoing EnMT.

4. Modulation of vascular property during metastasis

For metastasis to occur, cancer cells must leave the primary tumor, enter and exit the circulation, and then colonize distant sites. Vascular endothelial layer forms an important barrier for the cancer cells to leave the tumor (intravasation) and gain entry into the prospective metastatic organ (extravasation). This process of crossing the endothelial layer is termed as TEM and is the main crux of intravasation and extravasation processes [118]. Angiogenesis or formation of new blood vessels is one of the hallmarks of cancer [119]. Hypoxia, which is the main inducer of EMT [120], is also an important microenvironmental factor that initiates angiogenesis [121]. The blood vessels that arise in the tumor are typically immature and the ECs lack proper junctional contact. They have abnormal pericyte coverage that renders the blood vessels leaky and vulnerable. All these abnormalities in tumor blood vessels allow the cancer cells to intravasate relatively easily [122,123]. It has been hotly debated whether cancer cells are passively shed into the circulation due to defective vessels or enter the blood stream through an active mechanism [124]. In favor of

the latter, factors such as TGF β [125], Vascular endothelial growth factor (VEGF) [126–128], Angiopoietin-2 (Angpt2) [129], or Stromal derived factor-1 α (SDF-1 α) [130] have been shown to facilitate intravasation and are critical for extravasation as well. Because vasculature in distant organs is intact and functionally normal, it is highly likely that the process of extravasation is an active process and needs the assistance of growth factors and vascular permeability regulators to facilitate their TEM. A notable difference between intravasation and extravasation is that intravasation mostly involves abnormal tumor vasculature whereas extravasation targets normal blood vessels. The process of intravasation remains poorly investigated [131–133], but extravasation has been extensively studied. Extravasation occurs in two main steps. 1. *Adhesion of cancer cells to the endothelial layer*, 2. *Migration of the cancer cells across the endothelial layer*. The process of extravasation is often compared with leukocyte migration [134,135]. Some of the adhesion molecules and chemokines are commonly involved in TEM by both leukocytes and cancer cells [134,136–138]. Hence, the description of different steps in extravasation of cancer cells also gives a glimpse of leukocyte migration during immune reactions.

4.1 Adhesion of cancer cells to endothelial layer

Once in circulation, cancer cells are either passively carried away by the blood flow or acquire directional migration cued by growth factor or chemokine gradients [124,139,140]. Extravasation occurs after cancer cells physically get trapped or arrested in the small capillaries. This detainment of cancer cells in the capillaries facilitates the adhesion process of the cancer cells to the endothelium [12,141,142]. However, for stable attachment of cancer cells to endothelium and eventual extravasation, a wide range of ligand–receptor interactions are required, which involve selectins, cadherins, integrins, and immunoglobulins [118] (Table 2).

4.1.1 Selectins—Different selectins like E-selectin (CD62E), L-selectin (CD62L) and P-selectin (CD62P) have been implicated in tethering and rolling of cancer cells on ECs [143]. However, the rolling behavior of cancer cells has been exemplified only *in vitro* [144,145] and so far there is no *in vivo* supporting evidence. Nonetheless, E- and P-selectin have been shown to be important in colon and breast cancer metastasis *in vivo* [146,147]. In addition, the metastatic behavior of small cell lung carcinoma cells (SCLC) *in vivo* correlates with E-selectin-mediated adhesive and rolling events exhibited *in vitro* [144]. Furthermore, the metastatic event is reduced by 50% when SCLCs are xenografted in E- and P-selectin-deficient mice in comparison to wild type mice [148].

Under physiological conditions, E-selectin is not expressed on ECs. During cancer progression, inflammatory cytokines secreted by cancer cells can induce the expression of E-selectin on ECs and facilitate the adhesion of cancer cells to the endothelium [118]. Moreover, depending on the cancer type, cancer cells also express various selectin-specific ligands like HCELL (a CD44 glycoform), Carcinoembryonic antigen (CEA, CD66), podocalyxin-type protein-1 (PCLP-1), Mac-2bp, MUC-1, CD43, and P-selectin glycoprotein ligand 1 (PSGL-1) to bind to the E-selectin expressed on inflamed endothelium [149]. The protein and lipid scaffolds of these functional selectin-ligands are adorned with carbohydrate motifs, mainly tetrasaccharide sialyl Lewis x (sLe^x) and its stereoisomer sialyl Lewis a

(sLe^a) that form the minimal recognition moieties for selectins to bind [150]. These cancer cell surface glycans on selectin-ligands could also be used as diagnostic and therapeutic targets [151].

4.1.2 N-cadherin—Neuronal cadherin (N-Cadherin) plays an important role in cancer cell adhesion to ECs during extravasation. In 1984, N-Cadherin was identified as a molecule localized at the adherens junctions [152]. This cell surface presentation on ECs explains the interaction that is mediated by N-cadherin between cancer cells and ECs. N-cadherin typically forms homotypic interactions [153]. However, heterotypic interactions are also described [154]. Epithelial cancer cells typically express little or no N-cadherin. However, N-cadherin expression is upregulated during EMT, and the E-cadherin to N-cadherin switch is a hallmark of EMT. N-cadherin-mediated cancer cell-EC adhesion is a best example of heterotypic cell-cell interaction. In support of this adhesion process, several studies have revealed the role of N-cadherin in cancer cell-EC adhesion during extravasation. Interaction of melanoma cells with ECs is blocked upon the use of neutralizing N-cadherin antibody [155] or anti-sense RNA-mediated repression of N-cadherin [156]. This N-cadherin-mediated adhesion activates downstream effectors like Src kinase and β -catenin signaling in ECs, which further facilitate TEM during extravasation [156,157].

4.1.3 Integrins—Integrins, the α/β heterodimeric cell surface receptors, also play a critical role in adhesion of cancer cells to endothelium at the site of extravasation. Integrin VLA-4 on melanoma cells is shown to interact with VCAM-1 on endothelium, thus mediating its adhesion and TEM [158,159]. Furthermore, neuropilin-2 (NRP-2), a multifunctional nonkinase receptor for semaphorins expressed on renal carcinoma and pancreatic cancer cells, interacts with endothelial $\alpha 5$ integrin to mediate vascular adhesion and extravasation [160]. In prostate cancer metastasis to bones, adhesion of E-selectin ligands as well as $\beta 1$ and $\alpha V\beta 3$ integrins is sequentially required for extravasation [145]. Integrins $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha 5\beta 1$, $\alpha 6\beta 4$ expressed on tumor cells correlate with metastatic progression in melanoma, breast carcinoma, pancreatic, lung and prostate cancer [161,162], further validating the role of integrins in adhesion and extravasation.

4.1.4 Immune cell mediated adhesion—In addition to various cell adhesion molecules, special cells like leukocytes (including macrophages and platelets) further augment the process of cancer cell adhesion and extravasation. These cells act as linkers to promote the adhesion between cancer cells and endothelia. For instance, ICAM-1 is expressed on ECs, but MDA-MB-468 breast carcinoma cells lack its ligand $\beta 2$ -integrins. Neutrophil granulocytes, which express $\beta 2$ -integrins, behave as a bridge to mediate the interaction of MDA-MB-468 to the pulmonary endothelium [163]. Additionally, circulating melanoma cancer cells secrete high levels of interleukin-8 (IL-8) to attract neutrophils and upregulate their expression of $\beta 2$ -integrins, thereby adhering to endothelium through the neutrophils [164]. Similarly, platelets promote the interaction between cancer cells and activated endothelium through P-selectin or platelet integrin $\alpha IIb\beta 3$ [165–167]. Macrophages also behave as mediators in tumor cell-endothelial interaction. Macrophages produce cytokines, such as IL-8 and tumor necrosis factor- α (TNF- α), to stimulate endothelin (ET) and ET receptor (ETR) expression on breast cancer cells and human

umbilical vein ECs (HUVECs). The ET axis induces integrins and their counterligands on breast cancer cells and HUVECs, thus facilitating the adhesion process of cancer cells to HUVECs and also the TEM [168]. It has also been reviewed that leukocyte-cancer cell fusion facilitates chemotactic motility and metastasis [169,170].

4.2 Transendothelial migration (TEM)

As an initial step of extravasation, circulating cancer cells are arrested and tethered to the vasculature at the site of extravasation. Subsequently, cancer cells migrate through the endothelial layer of the blood vessel to eventually colonize the secondary site. Therefore, hyperpermeable vasculature facilitates TEM. The imperative role of vascular permeability in metastasis was established by using the bleomycin vascular damage model. Increased lung metastasis was observed upon bleomycin-induced damage of pulmonary endothelial layer [203–205]. TEM can occur in two ways. 1. Paracellular migration, where cancer cells cross the endothelial layer by disrupting the junctions between adjacent ECs and 2. Transcellular migration by which cancer cells cross the endothelial barrier by traversing through the EC body. Cancer cells usually take the paracellular route compared to transcellular mode of migration [118]. However transcellular migration has also been reported, where colon and breast cancer cells are shown to traverse through the ECs [206,207].

Under physiological conditions, the vessels are very stable and do not allow the passage of any kind of cells across them. However, during cancer metastasis, the ECs at the site of extravasation are activated as cancer cells secrete certain growth factors and cytokines such as VEGF, Angpt2, Angiopoietin-like 4 (ANGPTL4), Hepatocyte growth factor (HGF) and SDF1 α to permeabilize the vasculature (Table 3). An *in vitro* study highlights the importance of cancer cell-secreted factors, as treatment of ECs with conditioned medium (CM) obtained from pancreatic cells increases the intercellular junctional transport [208]. Here we discuss several cancer cell-secreted factors that facilitate TEM in detail.

4.2.1 VEGF—VEGF plays a critical role in the regulation of endothelial functions, such as angiogenesis, and vascular permeability. Owing to its permeability-inducing nature, it is also referred to as vascular permeability growth factor [209]. VEGF secreted by leukemic cells has been hypothesized to increase vascular permeability of blood brain barrier (BBB) [210]. VEGF signaling through VEGFR2 loosens the cell-cell contacts in established vessels [211]. Furthermore, VEGF modulates vascular permeability to allow the transendothelial passage of MDA-MB-231 breast cancer cells [127]. VEGF secreted by cancer cells or associated macrophages disrupts EC junction by interfering with VE-cadherin– β -catenin complex [212,213]. This disruption is mediated through Src or Yes, where the metastatic potential of VEGF-expressing cancer cells is decreased when they are injected intravenously into Yes-deficient mice [213]. Moreover, genetic or pharmacological inhibition of FAK in ECs prevents VEGF-stimulated permeability *in vivo*, highlighting the role of FAK in VEGF mediated vascular permeability [214]. Although VEGF has been shown to promote vascular permeability in several cases, most studies have employed exogenous source of VEGF or cancer cells with overexpression of VEGF. So it is still questionable if the endogenous amount of VEGF secreted by cancer cells is sufficient to promote TEM. It is likely that

VEGF may act with other permeabilizing factors to produce an additive or synergistic effect on vascular permeabilization and thus extravasation.

4.2.2 Angpt2—During vessel maturation, perivascular cells secrete Angpt1, which binds to the Tie-2 receptor on the surface of ECs. Activation of the Tie-2 signaling by Angpt1 stabilizes EC junctions and enhances vascular integrity [215]. ECs express Angpt2. However, Angpt2 functions by antagonizing Angpt1/Tie-2 signaling in ECs and negatively regulates the integrity of blood vessels [215]. Loss of blood vessel integrity caused by Angpt2 increases vascular permeability and facilitates TEM of cancer cells and consequently, metastasis [216]. Thus Angpt2 is implicated in cancer cell extravasation and metastasis. Angpt2 blockade has been shown to be associated with stronger EC-cell junction and reduced lung metastasis in melanoma [217], indicating the involvement of Angpt2 in vascular permeabilization. Furthermore, VEGF activates a calcineurin-NFAT pathway that increases Angpt2 transcription in the lung ECs and promotes the pulmonary metastasis [218,219]. Inhibition of Tie-2 signaling by Angpt2 renders the endothelium more responsive to inflammatory stimuli [220], thereby activating the endothelium to facilitate TEM. Angpt2 levels in patient serum display strong association with malignant tumor progression and survival [221,222].

4.2.3 Angptl4—Angptl4 undergoes proteolytic cleavage to form amino-terminal Angptl4 (nAngptl4) and carboxyl terminal Angptl4 (cAngptl4). This cleavage is tissue-dependent and is an important determinant of Angptl4 function. ECs primarily produce cAngptl4 that plays a major role in vascular permeability and angiogenesis [223]. However, the role of Angptl4 in vascular permeability is highly controversial. Angptl4 has been shown to prevent vascular leakiness induced by VEGF [224]. In another study, Angptl4-expressing tumor cells display reduced migration, invasion, and adhesion compared to control cells. Moreover, through its action on both vascular and tumor compartments, Angptl4 prevents metastasis by inhibiting vascular activity [225]. By contrast, TGF β in primary tumor microenvironment has been shown to induce Angptl4 expression in the departing tumor cells, which increases the permeability of lung capillaries by disrupting the EC-cell junctions and facilitates TEM [226]. Angptl4 is also a hypoxia-inducible gene [227]. During retinal angiogenesis, hypoxic Müller cells secrete Angptl4 to promote vascular permeability [228]. The function of Angptl4 does not involve Tie-2. It has been demonstrated that cAngptl4 disrupts vascular integrity by binding and activating integrin $\alpha 5\beta 1$ -mediated Rac1/PAK signaling and subsequently declusters VE-cadherin and claudin-5, leading to increased vascular permeability and lung metastasis [229]. In pursuit of Angptl4 being a vascular permeability factor, several other studies in esophageal squamous cell carcinoma, colorectal cancer and Kaposi's sarcoma have exemplified the pro-metastatic effect of Angptl4 [230–232]. Despite these extensive studies, further investigation on mechanisms involved in the diverse effects of Angptl4 on vascular permeabilization is necessary to unravel the discrepancy.

5. Why might an understanding of EMT-cancer cell and vasculature relationship be important clinically?

EMT and vascular modulation are two processes that are critical for successful metastasis. Various regulatory pathways of the two processes constantly crosstalk (Fig. 1). Inhibition of pathways involved in one process may activate the other, facilitating the events of metastasis. This could be exemplified by anti-angiogenic treatment, a commonly used adjuvant therapy for tumor growth inhibition. Anti-angiogenic therapy may have a devastating effect on metastatic progression. Treatment with tyrosine kinase receptor inhibitors increases metastasis in tumor models of animals [241,242]. Anti-VEGF treatment increases tumor cell invasion in glioblastoma [243]. Consistently, usage of Imatinib (a PDGFR β inhibitor) and Sunitinib (a VEGFR and PDGFR β inhibitor) as anti-angiogenic therapies reduces the pericyte coverage, leading to hypoxia-induced EMT and increased metastasis [63]. The observation that anti-angiogenic therapy promotes EMT in cancer cells underlines the importance of targeting vasculature in conjunction with inhibiting EMT and its associated events. In this regard, it is reasonable to target both angiogenesis and EMT. Indeed, inhibition of Casein Kinase 2 (CK2) blocks both EMT and angiogenesis. It has been shown that CK2 inhibitor blocks HIF1 α transcription in cancer cells, resulting in reduced EC migration and tube formation [244]. Another study shows reduced migration and invasion of A549 lung cancer cells upon CK2 inhibition [245]. Several other molecules and pathways are also shared by both angiogenesis and EMT, which include HIF1 α [62,246], SLUG [19,247], IL27 [248], and Bcl2-associated athanogene 3 (BAG3) [249]. Targeting these pathways may prove highly beneficial to combat metastasis. While anti-angiogenic treatment increases the occurrence of EMT, therapies targeting vascular adhesion molecules such as integrins and selectins may inhibit the extravasation process of EMT-cancer cells, thus minimizing the incidence of metastasis. With ongoing research advancing our understanding of metastasis, it may not be too far when many of the mechanistic mysteries will be solved and successfully translated into the clinics for effective anti-metastatic therapy.

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Highlights

A variety of signaling pathways induce EMT in cancer cells

EMT facilitates cancer cell invasion and migration to blood vessels for intravasation

Cancer cells use adhesion molecules to attach to endothelium for extravasation

Cancer cells secrete vascular permeabilizing factors to overcome endothelial barrier

Figure 1. Signaling pathways and mediators involved in the process of EMT and vascular modulation

Various signaling pathways such as Wnt, Notch, TGF β , HIF1 α , Hedgehog, and PI3K induce EMT and facilitate the migration and invasion of cancer cells, which are further enhanced by proteases like Cathepsins and Matrix metallo proteinases (MMPs). EMT-cancer cells that have moved towards the vasculature may passively transmigrate through the endothelia due to the highly leaky nature of tumor vasculature. These EMT-cancer cells then migrate through the blood stream to distant organs, where they adhere to the vascular endothelia through molecules like Integrins, N-cadherin and Selectins. Certain immune cells behave as anchors to facilitate the adhesion of cancer cells to endothelial cells. Following adhesion, cancer cells actively transmigrate through the endothelia by secreting growth factors and cytokines including VEGF, Angpt2 and Angptl4 that increase the permeability of the vasculature. TEM: Transendothelial migration.

Table 1

Signaling pathways that promote EMT

Signaling pathway	References
TGF β	[55–58]
HIF1 α	[69,70]
Wnt	[5,19,86]
Notch	[5,85]
Hedgehog	[87–90]
Ras	[16,91]
Src	[92,93]
PI3K/ AKT	[94]
JAK/STAT	[95]
BMP	[96]
FGF	[97]
EGF	[98]

Table 2

Cell adhesion molecules (CAM) that facilitate metastasis

CAM Family	Name	References
Selectins	E-Selectin	[171,172]
	P-Selectin	[173,174]
	L-Selectin	[150,175]
Integrins	$\alpha 4\beta 1$ (VLA4)	[176–179]
	$\alpha 6\beta 1$ (VLA6)	[180,181]
	$\alpha v\beta 3$	[182]
	$\alpha v\beta 5$	[183]
	$\alpha 5\beta 1$	[184]
	$\alpha 6\beta 4$	[185,186]
	$\alpha IIb\beta 3$	[171,187]
	$\beta 1$	[188,189]
Cadherins	P-Cadherin	[190–192]
	N-Cadherin	[156,191,193]
Immunoglobulin Superfamily CAM	Vascular Cell Adhesion Molecule (VCAM)	[194]
	Intercellular CAM (ICAM)	[195,196]
	Melanoma CAM (MCAM)	[197,198]
	L1CAM	[199,200]
	Neural CAM (NCAM)	[201,202]

Table 3

Vascular permeability factors that assist TEM

Permeability Factors	References
VEGF	[118,128]
TGF β	[118,226]
Angpt2	[233,234]
Angptl4	[226,235,236]
HGF	[118,237]
SDF1 α	[238,239]
Cdc42	[118,240]