

Tumor cell intravasation

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Chiang SP, Cabrera RM, Segall JE. Tumor cell intravasation. *Am J Physiol Cell Physiol* 311: C1–C14, 2016; doi:10.1152/ajpcell.00238.2015.—The process of entering the bloodstream, intravasation, is a necessary step in the development of distant metastases. The focus of this review is on the pathways and molecules that have been identified as being important based on current in vitro and in vivo assays for intravasation. Properties of the vasculature which are important for intravasation include microvessel density and also diameter of the vasculature, with increased intravasation correlating with increased vessel diameter in some tumors. TGF β signaling can enhance intravasation at least in part through induction of EMT, and we discuss other TGF β target genes that are important for intravasation. In addition to TGF β signaling, a number of studies have demonstrated that activation of EGF receptor family members stimulates intravasation, with downstream signaling through PI3K, N-WASP, RhoA, and WASP to induce invadopodia. With respect to proteases, there is strong evidence for contributions by uPA/uPAR, while the roles of MMPs in intravasation may be more tumor specific. Other cells including macrophages, fibroblasts, neutrophils, and platelets can also play a role in enhancing tumor cell intravasation. The technology is now available to interrogate the expression patterns of circulating tumor cells, which will provide an important reality check for the model systems being used. With a better understanding of the mechanisms underlying intravasation, the goal is to provide new opportunities for improving prognosis as well as potentially developing new treatments.

intravasation; metastasis; TGF β ; EGFR; uPA; angiogenesis

THE METASTATIC CASCADE involves a number of steps including invasion, intravasation, and extravasation, leading to growth at a secondary site. Tumor cells invade into normal tissue, often towards lymphatic or blood vessels. Upon reaching the vessel, these cells must then cross the endothelial barrier and enter the circulation. In the circulation, tumor cells may be killed by shear stress and the immune system, or become lodged in a distant capillary bed, where they can undergo extravasation and form a secondary tumor. Although the metastatic process is very inefficient, with only about 0.01% of circulating tumor cells actually leading to the formation of tumors at secondary sites, it is important to study the process of intravasation, as the rate at which tumor cells gain access to the peripheral circulation can affect the efficiency of metastasis.

There are many factors that contribute to cancer cell intravasation, including other cells in the tumor microenvironment, proteases, signaling molecules, and environmental conditions at the tumor and associated vasculature. These cells and molecules play important roles in allowing the tumor cells to invade through the basement membrane, adhere, and pass through the endothelial cell junctions in order to enter the circulation. A number of in vivo and in vitro systems have been developed to study intravasation, and they have been useful in identifying the roles of specific cells and molecules in enhancing intravasation. A number of earlier reviews have discussed

intravasation in cancer (10, 31, 32, 110, 121, 126). The focus of this review will be on what has been learned regarding the mechanisms contributing to intravasation.

A clearer understanding of intravasation may provide opportunities for developing new prognostic markers as well as therapeutic options for preventing the spread of metastases. One of the complexities of studying intravasation in cancer, however, is the wide variation in properties of different tumors. Thus different tumors are likely to utilize different mechanisms for intravasation. Successful development of new markers or treatments will need to go hand in hand with identifying the appropriate tumors for which they can be used.

Glossary

ANGPTL4	Angiopoietin-like 4
API	Activator protein 1
CAF	Cancer-associated fibroblast
CAM	Chorioallantoic membrane
CDCP1	CUB domain-containing protein 1
CSF1R	Colony-stimulating factor 1 receptor
CTCs	Circulating tumor cells
CTGF	Connective tissue growth factor
CXCR1,2	Chemokine (C-X-C motif) receptor 1, 2
CYR61	Cysteine-rich angiogenic inducer 61
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FAK	Protein tyrosine kinase 2 (PTK2) Focal adhesion protein

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FGF1	Fibroblast growth factor 1
HBEGF	Heparin-binding EGF
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HMGA2	High mobility group AT-hook 2
HOXD10	Homeobox D10
ICAM1	Intercellular adhesion molecule 1
IFP	Interstitial fluid pressure
IL-6	Interleukin 6
IL-11	Interleukin 11
LHX2	LIM homeobox 2
LIMK	LIM domain kinase
MIW	Mammary imaging window
MMP	Matrix metalloproteinase
N-WASP	Wiskott-Aldrich syndrome-like (WASL)
PAR1	Protease-activated receptor 1
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
PDCD4	Programmed cell death 4
PI3K	Phosphoinositide 3-kinase
PTHrP	Parathyroid hormone-related protein
PTK2	Protein tyrosine kinase 2
PVT	Prevascularized tumor
RHOA	ras homolog family member A
S1P	Sphingosine 1 phosphate
SERPINE	Serpin peptidase inhibitor, Clade E
SLPI	Secretory leukocyte peptidase inhibitor
SNAIL	Snail family zinc finger 1
SPINK1	Serine peptidase inhibitor, Kazal type 1
SRC-1	Steroid receptor coactivator-1
STAT3	Signal transducer and activator of transcription 3
STC1	Stanniocalcin 1
SULF2	Sulfatase 2
TGFB	Transforming growth factor- β
TMEM	Tumor microenvironment of metastasis
TNFA	Tumor necrosis factor- α
uPA	Urokinase type plasminogen activator
VEGF	Vascular endothelial growth factor
VIM	Vimentin
ZEB1,2	Zinc finger E-box binding homeobox 1,2

Intravasation by Nontumor Cells

Although this review is on intravasation by cancer cells, intravasation is a naturally occurring process that is important during development, as well as for a normally functioning immune system. Signals such as growth factors or tissue injury can guide populations of cells through normal tissue, and can lead them into crossing the endothelial barrier and into vessels. The crossing of these cells maintains vessel structure and integrity by retaining cell junctions and keeping vessels from leaking, unlike tumor cell intravasation which may in some tumors be accompanied by loss of endothelial cells and disrupted cell junctions.

Entry into the blood from the bone marrow requires intravasation by developing immune cells. Depending on the circumstances, immune cells may cross via paracellular (through the cell) or transcellular (between cells) diapedesis (79, 103). During maturation in the thymus, T-cells go through a process of selection, eventually leading to egress from the thymus and entry into circulation. From there, they become activated by

antigens in lymphoid organs, and again must enter the circulation to reach sites of infection (72). Egress from the thymus and lymphoid organs requires the S1P receptor S1P₁, a factor also important for proper endothelial cell function (104).

Hematogenous vs. Lymphatic Intravasation

Intravasation of tumor cells into the circulation can occur through entry into blood vessels or lymphatic vessels, with the majority of entry occurring through hematogenous (blood vessel) routes. The process of intravasation is very inefficient, and the shear stress of blood flow alone may be enough to destroy many of the cells entering into the bloodstream. Once in the bloodstream, the cancer cells must also be able to evade the immune system and implant at a secondary site to form distant metastasis.

Hematogenous intravasation can be an active or passive event, depending on the tumor type, blood vessel structure, and conditions occurring in the tumor microenvironment. During intravasation, there are a number of changes that occur within the tumor cells themselves, as discussed in the sections below. It has been seen that tumor cells can undergo changes in cytoskeletal activity due to sensing a chemoattractant gradient. There is also evidence of the upregulation of integrins and other adhesion molecules in order to facilitate attachment of tumor cells to endothelial cells (31, 62).

Lymphatic intravasation (entry into lymphatics) is another pathway by which tumor cells can enter the circulation. After converging on the major thoracic duct, lymph vessels eventually drain into the blood, thereby indirectly allowing tumor cells to gain access to the venous blood supply. However, along the way, the tumor cells encounter a series of lymph nodes, which are often the first sites of metastasis observed for a number of cancers. These sentinel lymph nodes are the ones that are closest to the site of the primary tumor, and are resected and biopsied in order to check for early signs of metastasis.

Whether a tumor cell undergoes hematogenous or lymphatic intravasation is dependent on a number of factors (128). One obvious factor could be tumor cell accessibility to vessels. Tumor angiogenesis leads to a network of microvasculature that is then available for access to tumor cells, as discussed below. It has also been seen that tumors undergo lymphangiogenesis, but that these lymph vessels within the tumor may not function as well as those formed on the outside of the tumor tissue (83). The relative amount of hematogenous versus lymphatic intravasation may depend on the relative proportions of blood vessels and lymphatics induced by the tumor.

The differences in structure between blood vessels and lymphatics likely lead to differences in the mode of intravasation. Structurally, lymphatics do not have the tight endothelial junctions seen in blood vessels. Entry into lymphatics may be limited by the ability of tumor cells to invade through intervening connective tissue, with the actual entry into the lymphatics occurring with relative ease. Additionally, the flow in lymphatic vessels is far less than that in blood vessels, making survival in the lymphatics easier, due to decreased shear stress (2). Thus the presence of metastases in local lymph nodes may reflect the ability of tumor cells to penetrate connective tissue barriers. However, the step of penetrating the blood endothelial cell barrier may require capabilities in addition to simple

invasion. Therefore, the importance of the lymphatic route for development of distant metastasis is open to debate. There is not a strict correlation between development of distant metastases and the presence of a chain of lymph nodes with tumor cells going to the major thoracic duct (107). It is likely that distant metastases are initiated by direct intravasation into the blood supply either in the primary tumor itself, or potentially in the lymph nodes. The focus of this review is on mechanisms of entry into blood vessels.

In Vivo Models

There are two *in vivo* common measures of intravasation. The direct measure of intravasation is the measurement of circulating tumor cells. This measurement assumes that there is fairly rapid filtration of tumor cells by capillary beds, especially the lungs for cells entering the venous system, and is supported by published data (34, 51). The intravasation efficiency is then inferred from the number of circulating tumor cells. Comparisons should ideally be done with tumors of similar size and vascular density in order to be focused on intravasation. Another direct method which is technically more challenging is to directly image cells protruding through the blood vessel wall (46, 112). An indirect but simpler method is to measure metastatic burden generated by a primary tumor. Because this "spontaneous metastasis" measurement can be influenced by the ability of cells to extravasate and survive in the target organ, an accompanying "experimental metastasis" measurement should be made in parallel, in which the metastatic efficiency of cells directly introduced into the vasculature by *iv* injection is also determined. Multiple *in vivo* models have been developed to study the intricate, multistep process of tumor metastasis, including chick embryo, fluorescent transgenic zebrafish, and murine models. Each model system provides unique advantages that have been utilized to investigate and visualize tumor cell intravasation as well as the interactions between tumor and stromal cells during this event.

The chick embryo is naturally immunodeficient, which allows human tumor cells to be transplanted onto the chorio-allantoic membrane (CAM) without risk of immunological rejection. Furthermore, the CAM is extensively vascularized and located directly underneath the eggshell and is thus easily accessible for manipulation and visualization. A detailed review covers the various experimental approaches using the CAM model (28). Palmer et al. (84) provide methods for the spontaneous metastasis model, which involves exposure of the CAM and subsequent engraftment of the tumor cells onto the membrane. Quantitative analysis of what are termed intravasated cells is performed by a real-time PCR amplification of human-specific Alu sequences (144) that was first developed by Kim et al. (61). This is an indirect measurement of metastasis, as it does not distinguish between circulating tumor cells in the vasculature and those that have arrested and possibly proliferated in target organs.

The process of intravasation can also be visualized using fluorescent transgenic zebrafish (77). Zebrafish are cost-effective in maintenance and optically transparent, which allows noninvasive, high-resolution imaging of tumor progression. Using this model, it was shown that RhoC, a regulator of the actin cytoskeleton, induces cytoskeletal changes to mediate intravasation (112). In this study, tumor cells were injected into

the zebrafish peritoneal cavity, allowing them to invade into the tissues, remodel the vasculature, and form microtumors. The intravasation events were then visualized by confocal microscopy. Tumor cells expressing RhoC formed large membrane extensions that protruded through VEGF-induced vascular openings into the vessel lumen.

Visualization and quantitation of intravasation can also be performed in mouse models of cancer. In a murine model of breast cancer, tumor cell intravasation was directly visualized using intravital multiphoton imaging of fluorescently labeled tumor cells (46, 130). Intravasated tumor cells can be quantified using whole blood drawn from the right ventricle before the blood perfuses the pulmonary vasculature, showing that the number of CTCs correlates with lung metastasis (129). Furthermore, two patterns of tumor cell migration were identified by multiphoton intravital imaging: single cells and multicellular streams, with the multicellular streams correlating with intravasation (86). Using a Mammary Imaging Window (MIW) combined with fluorescent photoconversion, trafficking of cells can be followed over a week (56). Using this method, it was shown that vascular microenvironments promote tumor cell intravasation (42). Spontaneous lymphogenic metastasis of metastatic breast tumor cells has also been investigated *in vivo* through high-resolution, noninvasive fluorescence imaging (25, 47). The presence of high interstitial fluid pressure (IFP) in the tumor and fast drainage rates of the FITC-dextran into the tumor-associated lymphatic vessels suggest that lymphatic intravasation can be induced by high IFP (92).

In Vitro Models

In vitro systems such as microfluidic devices, artificial microvessels, and Transwell assays have been developed to mimic the pathophysiology of a tumor microenvironment. Microfluidic models provide a basis for studying complex cell-cell interactions within a 3D microenvironment (113). A single microfluidic chip that integrates intravasation and extravasation was used to study LOVO tumor cells and found that tumor cells degraded the ECM by the expression of MMPs. Thus, this system can be utilized to evaluate MMP inhibitors and drug candidates for intravasation (106). Zervantonakis et al. (139) designed a microfluidic-based assay that reconstructs the tumor-vascular interface in 3D and integrates it with real-time imaging and measurement of the endothelial barrier function. Specifically, this assay consists of two independent micro-channels that are interconnected via a 3D ECM hydrogel. Tumor and endothelial cells are seeded in each micro-channel and additional cell types may be added to model heterotypic cell-cell interactions during intravasation. Using this assay, the authors showed that macrophage-secreted TNFA increases endothelial permeability and intravasation rate.

Tumor-vascular interactions have also been investigated using an artificial microvessel system integrated with live-cell fluorescence microscopy (127). A perfusable artificial vessel is lined with an endothelial monolayer in a type I collagen matrix, and fluorescently labeled tumor cells are placed in the ECM around the vessel. Using this platform, it was found that during an intravasation event a single tumor cell forms membrane extensions into the lumen of the microvessel, disrupting the

monolayer, and can leave a portion of its cytoplasm in the ECM as it enters the microvessel.

Transendothelial migration of tumor cells can also be quantified using a Transwell assay that measures movement of tumor cells from the basal to apical side of an endothelial monolayer (99). In this system, the endothelial monolayer is plated on the underside of a Transwell and establishes the appropriate polarity such that tumor cells and macrophages added to the interior of the Transwell face the basal side of the monolayer, mimicking intravasation. Imaging of the tumor cell transendothelial migration in this assay revealed that tumor cells preferentially cross the endothelial barrier via paracellular migration while in close association with macrophages (Fig. 1).

Angiogenesis

Intravasation is dependent on the access of tumor cells to vasculature, and thus tumors with more vasculature would be expected to have an increased intravasation rate, all other factors being equal. Consistent with this prediction, in colorectal cancer the density of intratumoral microvessels was found to be a predictive factor for the presence of circulating tumor cells (117, 133). Expression of the angiogenic factor VEGF correlated with the presence of liver metastases. Inhibition of VEGF using a functional blocking antibody was found to reduce microvessel density and intravasation of prostate cancer cells in the CAM model (22). Similarly, in the PyMT breast cancer model using intravital imaging, bursts in vascular permeability correlated with intravasation events, and both phenomena were inhibited by a VEGF blocking antibody (46). Fibroblast growth factors are also angiogenic factors (96), and expression of FGF1 in MCF7 breast cancer cells resulted in increased blood vessel density in parallel with increased intravasation frequency (141). However, these cells were unable to form macrometastases in the lung and seemed to be unable to extravasate and proliferate before eventually being cleared. On the other hand, a conflicting report concerning VEGF expression and microvessel density (120) in colorectal cancer showed metastasis associated with a lower VEGF-positive cell count and a decrease in microvessel density. However, metastasis did correlate with an increase in microvessel diameter, which is consistent with other reports supporting the importance of vessel diameter for intravasation.

Thus the specific characteristics of the blood vessels may influence the mechanism by which intravasation occurs. Yamamura et al. (133) compared the diameter of the microvessel lumens in the primary tumor tissues of patients with or without metastatic disease and found that patients with metastatic disease had significantly larger microvessels in the primary tumors. The presence of dilated sinusoidal vessels in the tumor may allow formation of tumor emboli in an invasion-independent manner (114, 115). In a renal cancer model, the majority of cells leaving the primary tumor were nonviable, consistent with a passive shedding mechanism (10, 11). Emboli surrounded by endothelium have been observed in the veins of renal cell carcinoma, hepatocellular carcinoma, and follicular thyroid carcinoma, suggesting a process where an endothelium-lined outpocketing of the tumor into the sinusoidal space could detach and enter the circulation through shearing. These emboli could have a higher efficiency of seeding distant sites (1, 34, 40, 68). Such tumor fragments have been found in the clear cell subtype of renal cell carcinoma to correlate with pulmonary metastases (55) and higher levels of VEGFA. Overexpression of VEGFA in a melanoma animal model was also shown to generate sinusoidal vessels and the presence of endothelium-coated tumor emboli (63). However, in the CAM model, expression of VEGF was found to enhance leakiness and intravasation in smaller diameter vessels (74).

Other perturbations of the vasculature can have complex consequences for intravasation. VEGFB expression can reduce blood perfusion efficiency, resulting in increased hypoxia, which can stimulate invasiveness. In parallel it can increase vessel leakiness, with the net result being increased intravasation and metastasis in tumors that grow less efficiently (135). Reduced expression of *Shb*, an adaptor protein downstream of VEGFR-2, can result in vascular leakiness and increased metastasis (138). Expression of the matrix metalloproteinase MMP-1 by tumor cells has also been shown to enhance vascular permeability and intravasation without affecting invasive properties of the tumor cells in the CAM assay, and a potential mediator of this effect is PAR1 (52). IL-6 and MMP-9 promote cancer metastasis by increasing resistance to apoptosis, enhancing angiogenesis, and accelerating degradation of the ECM (17, 29, 69). Conversely, activation of myosin light chain kinase in endothelial cells can be induced by

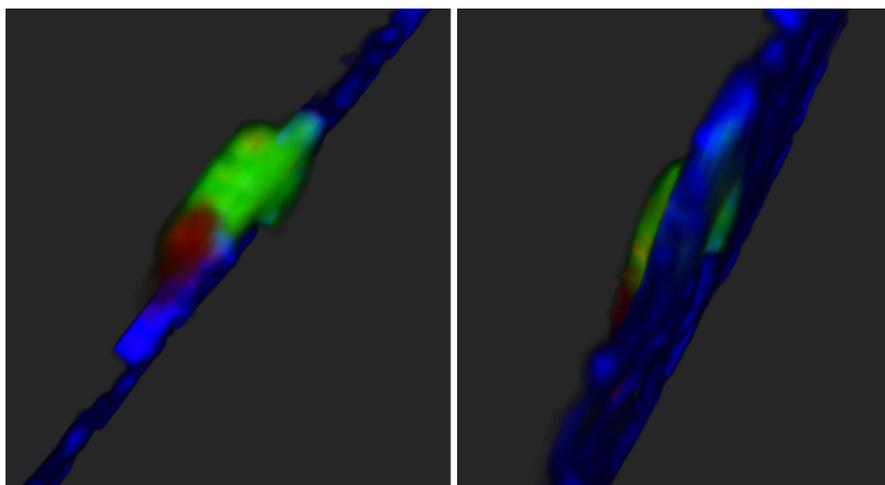


Fig. 1. Two views of an MDA-MB-231 breast cancer cell (green) crossing an endothelial cell layer (blue) in association with a macrophage (red) in the Transwell in vitro intravasation assay (99). The basal side of the endothelium is on the *right*.

intravasating tumor cells(60), leading to the activation of myosin II and inducing endothelial myosin contraction. Interestingly, inhibition of myosin regulatory light chain diphosphorylation led to a reduction in transcellular migration and an increase in paracellular migration. Thus, myosin light chain kinase expression in endothelial cells may play a key role in directing intravasation pathways.

An intriguing potential mechanism for intravasation involves vascular mimicry by tumor cells (105). Expression of the secreted proteins SERPINE2 or SLPI in mammary tumor cells increased their frequency of lining blood vessels in tumors without an identifiable endothelial surface layer, thus putting them in direct contact with blood. The expression of these proteins suppressed coagulation, likely enabling blood flow to be maintained and facilitating detachment of tumor cells from the main tumor body (122).

The vasculature has a major role in determining conditions within the tumor microenvironment including tumor hypoxia. The cell density at primary tumors, due to the limited blood supply, creates conditions where cells experience a shortage of nutrients and oxygen. These hypoxic conditions can induce the expression of VEGF, causing increased angiogenesis, lymphangiogenesis, and vessel permeability (12). Hypoxia can also increase tumor cell invasiveness by making tumor cells more sensitive to growth factors such as HGF (89, 116). This then allows tumor cells to invade through normal tissue, degrade the basement membrane, and eventually cross the endothelial barrier. Another factor regulated by hypoxia is the secreted protein angiopoietin-like 4 (ANGPTL4), which acts to disrupt endothelial cell junctions and therefore allows tumor cells to migrate into the vasculature. ANGPTL4 expression can enhance vascular permeability at the primary tumor and extravasation (49, 140); it would be interesting to test whether it plays a role in intravasation.

TGFB

A number of studies combine to make a strong case for TGFB signaling regulating intravasation in breast cancer and hepatocellular carcinoma. Muraoka et al. (80) utilized systemic long-term application of a soluble TGFB receptor fusion protein to sequester TGFB in the transgenic MMTV-PyMT breast cancer model and found suppression of circulating tumor cell numbers. Although the rate of tumor formation, vascularity, and tumor weight were not altered, treatment with the fusion protein resulted in more cystic morphology and increased apoptosis. Possible mediators of intravasation that were reduced included PI3K signaling, VIM, MMP-2, and MMP-9. A potential role for TGFBR2 in intravasation was shown by Keklikoglou et al. (58), who found that suppression of TGFBR2 expression inhibited intravasation of MDA-MD-231 breast cancer cells in the indirect CAM assay. The authors showed that expression of miR-373/520 family members also suppresses TGFBR2 expression as well as intravasation. Although miR-373 levels were too low to quantitate in patient tumors, a negative correlation was found between expression of miR-520c and TGFBR2 in ER-negative tumors, consistent with miR-520c regulating TGFBR2 levels in patients. TGFB target genes that were affected included ANGPTL4, PTHrP, and PAI-1.

Using a SMAD2-GFP expression construct to follow SMAD2 localization in the nucleus, and a CAGA12-CFP reporter for TGFB signaling, Giampieri et al. (38) visualized TGFB signaling in rat mammary adenocarcinoma MTLn3E cells undergoing single cell motility in vivo using intravital imaging. The authors also confirmed a role for SMAD signaling using suppression of SMAD4 expression. Entry into lymphatic vessels was not dependent on TGFB signaling, while overexpression of TGFB1 increased hematogenous intravasation. Intriguingly, overexpression of TGFB1 appeared to inhibit proliferation in the lung although it enhanced intravasation. Thus transient TGFB signaling at the intravasation step may be the most effective way for tumor cells to intravasate while not inhibiting their proliferation when arriving at a metastatic site. TGFB pathway activation in the primary tumor was found to be quite heterogeneous, suggesting the presence of transient signals being generated by specific local microenvironments within the tumor. Consistent with inhibition of metastatic growth by TGFB, overexpression of a downstream mediator of TGFB, p12^{CDK2-AP1}, stimulated EMT and intravasation of hamster cheek pouch carcinoma cells, but did not enhance overall metastasis (119). Intriguingly, the presence of tumor cells which can intravasate can enhance the metastasis of other cells in the same tumor, suggesting that in a heterogeneous tumor, cells which have undergone EMT may enable the intravasation of other cells which have not undergone EMT but are better able to seed distant sites (16, 35, 118).

A number of downstream genes have been identified that could mediate TGFB's contribution to intravasation. Patsialou et al. (87) found that TGFB stimulation results in increased expression of CSF1R in basal breast cancer cells. Suppression of CSF1R expression in the tumor cells resulted in reduced in vivo motility and intravasation. Echoing the result found by Giampieri et al. (38), CSF1R activity appeared to inhibit proliferation in vivo, in parallel with stimulating invasion and intravasation. CSF1R activity was found to suppress the expression of keratins and claudins, and overexpression of claudins resulted in reduced invasion, suggesting that maintenance of the claudin low state is supported by TGFB driven expression of CSF1R.

LHX2 has been identified as another downstream target of TGFB that can enhance intravasation and metastasis (64). High levels of LHX2 in the primary tumor correlated with metastasis and worse outcome in breast cancer patients. Increased expression of LHX2 as a transgene in the PyMT model resulted in increased intravasation and metastasis, with some increase in growth as well. Both invasion and vessel size are increased with expression of LHX2, and could result in the increased intravasation capability. A possible mediator of LHX2's effect is PDGFB.

A classic downstream mediator of TGFB signaling is the transcription factor Twist. Knockdown of Twist in the 4T1 breast cancer model did not affect primary tumor or anchorage independent growth, but did significantly reduce circulating tumor cells and metastasis (134). Twist expression was higher in more metastatic breast cancer cell lines, and also in lobular breast carcinoma, which is distinguished by its invasive character. A potential downstream mediator of Twist for intravasation in the 4T1 breast cancer model is miR-10b, which targets HoxD10, a suppressor of RHOC expression (70).

In hepatocellular carcinoma, the long noncoding mRNA lncRNA-ATB (lncRNA-AL589182.3, ENST00000493038) is upregulated by TGF β (137). LncRNA-ATB acts as a competing RNA for the miR-200 family, and by inhibiting miR-200 function, it stimulates the production of the miR-200 targets ZEB1 and ZEB2. Lnc-ATB also could enhance lung and liver colonization, but that was not through EMT and miR-200 regulation but rather through increased IL-11 production and STAT3 signaling, reiterating the separation between production of circulating tumor cells and enhanced growth capability at distant sites. Another TGF β target potentially important for intravasation in hepatocellular carcinoma is CTGF/CCN2. Suppression of CTGF expression results in reduced intravasation in the CAM assay in parallel with reduced fibrosis (73). It is possible that the paracrine stimulation of tumor-associated fibroblasts by CTGF mediates an enhancement of intravasation. The exact mechanism by which this occurs remains to be determined.

Receptor Tyrosine Kinases

The most detailed examination of receptor stimulation of intravasation has been based on the EGF receptor family. Although the EGFR family is associated with tumor growth, it also can have a significant contribution to invasion and intravasation. Overexpression of the EGFR was shown to affect in vivo invasiveness, intravasation, and metastasis without affecting primary tumor growth of MTLn3 cells in SCID mice (132). Intravasation was measured as the number of circulating tumor cells. Similar results were found for fibrosarcoma and head and neck cancer in the CAM assay (74). Since EGFR overexpression enhanced the ability of tumor cells to invade locally, the enhancement of intravasation could be due to enhanced ability to approach the blood vessel, or enhanced ability to cross the blood vessel barrier. Studies comparing EGFR inhibitors with ErbB2 inhibitors suggest that EGFR signaling may be more important for the approach to the vessel, while ErbB2 signaling may be more directly involved in the intravasation step (57). Similarly, overexpression of the EGFR ligand HBEGF was also able to stimulate in vivo invasion, intravasation and metastasis with little effect on primary tumor growth in MTLn3 cells and human MDA-MB-231 breast cancer cells (142). SPINK1 also appears to act as an autocrine EGFR ligand for prostate cancer cells independent of its protease inhibitor properties, and suppression of SPINK1 expression reduced intravasation in the CAM assay (4). The sensitivity of the EGFR to low levels of ligand can be enhanced by the actin-binding protein Mena/ENAH (100). Knockout of Mena expression results in reduced intravasation in the MMTV-PyMT breast cancer model (101), while overexpression of a particular isoform, termed Mena^{INV}, can increase intravasation together with invasion in response to EGF (90, 100). Other members of the EGFR family include ErbB2 and ErbB3, and the ErbB2/ErbB3 heterodimer, which is activated by heregulin, has been shown to enhance intravasation in SCID mice of MTLn3 and MDA-MB-435 cells (131). Factors that regulate expression of EGFR family members could also direct intravasation; a possible example is the steroid receptor coactivator-1 SRC-1. Knockout of SRC-1 resulted in reduced ErbB2 expression in the MMTV-PyMT mammary tumor model in parallel with reduced intravasation and metastasis and little effect on pri-

mary tumor growth (124). However, the SRC-1 phenotype is more complex—the grade of the primary tumor may be altered as well as expression of important factors such as CSF1 discussed below.

Downstream of the receptor, PI3K activity has been shown to contribute to intravasation. Knockdown of the endogenous p110 alpha catalytic subunit in MDA-MB-231 breast cancer cells and replacement with two mutants commonly found in cancer resulted in increased intravasation, with the helical domain mutation E545 producing the strongest enhancement (85). Mutation of the PI3K-binding sites on ErbB3 led to reduced PI3K activation, and corresponding reductions in invasion in vivo and intravasation in an in vitro intravasation assay (108). A potential downstream target of PI3K is the actin regulator N-WASP. Either expression of a dominant-negative form of N-WASP or shRNA knockdown resulted in reduced intravasation of MTLn3 breast cancer cells without affecting primary tumor growth (43). N-WASP is important for formation of invadopodia, localized F-actin-containing structures which cause localized matrix degradation (5, 45, 50), and invadopodia could enhance penetration of the endothelial basement membrane during intravasation. Intravital imaging and multiparametric analysis shows a correlation between invadopodia and intravasation (41). Similarly, an in vitro intravasation assay shows that macrophages can stimulate intravasation and that in parallel macrophages stimulate invadopodium formation and RhoA activity in MDA-MB-231 breast cancer cells (99).

A number of other regulators of invadopodium function have been found to contribute to intravasation. Arg is a tyrosine kinase that regulates cortactin activity in invadopodia, and suppression of Arg in MDA-MB-231 cells dramatically reduced invasion and intravasation in vivo, while remarkably increasing primary tumor growth and the ability of tail vein injected tumor cells to grow in the lung (39). This experiment emphasizes how invasion and intravasation can be separated from tumor growth, and is consistent with the observations noted above that tumor cells can decide to “go or grow.” Invadopodia are also regulated by integrins, and the ERM protein talin integrates adhesion signals to regulate intracellular pH through the sodium/proton antiporter NHE-1. Like Arg, suppression of talin results in reduced intravasation although primary tumor growth is stimulated (6). Cofilin actin-severing activity is regulated by intracellular pH, and thus the talin regulation of NHE1 activity also contributes to cofilin activity. An alternative mechanism of regulation of cofilin is through phosphorylation by LIMK, which suppresses its activity, and perturbation of LIMK activity also affects intravasation (125).

The ERK pathway also can contribute to intravasation. Expression of the Raf inhibitor RKIP is reduced in a number of tumors, and increased expression of RKIP in MDA-MB-231 breast cancer cells resulted in reduced invasion and intravasation in vivo without affecting tumor growth (26). More detailed analysis indicated that through regulation of the ERK pathway, RKIP affects a pathway involving myc, LIN28, let-7, and HMGA2, resulting in changes in expression of Snail and other proteins involved in tumor cell invasion as well as secretion of the chemokine CCL5, which may recruit and program tumor-associated macrophages (36).

Proteases

There is significant evidence for a role of the uPA/uPAR system in intravasation. Studies with the inoculation of HEp3 cells into the CAM demonstrated that uPA blocking antibodies could inhibit invasiveness and metastasis but not extravasation, arguing for a role in intravasation (82). Quantitation of tumor cell intravasation in the CAM assay using uPA mRNA knock-down was consistent with this possibility (61). uPA was also identified as important for invasion and intravasation by comparing high and low metastatic derivatives of the HT1080 fibrosarcoma cell line using a protease activity-based profiling method (71). Treatment with the uPA inhibitor PAI-1/SERPINE1 or a uPA blocking antibody, mAB-112(9), could block intravasation while addition of uPA could stimulate intravasation with limited effects on primary tumor growth. Similar results were seen with a prostate cancer cell line (7, 22). The microRNA miR-182 has been shown to stimulate intravasation in a mouse sarcoma model, and part of its effect could be through suppression of the uPA inhibitor PAI-1 (102).

Reciprocal activation of uPA and plasminogen is strongly enhanced by binding of uPA to its receptor on the cell surface, uPAR. The importance of uPA binding to uPAR was demonstrated through the use of peptides that were developed to bind to uPAR and compete with uPA (94). Addition of the antagonist peptide significantly reduced intravasation of HEp3 cells in the CAM assay compared with a scrambled peptide control. Similarly, uPAR-binding peptide antagonists inhibited plasmin activation by uPA. Expression of uPAR can be increased by src through enhanced API activity in colon cancer SW480 cells. Src expression stimulated invasion and intravasation, and the enhanced invasion was shown to be dependent on uPAR (66). Conversely, suppression of uPAR expression is a potential mechanism of action of the tumor suppressor PDCD4. PDCD4 expression was found to be inversely correlated with uPAR, and PDCD4 suppressed SP1-mediated activation of uPAR transcription. Overexpression of PDCD4 in HEp3 cells suppressed intravasation in the CAM assay (66). PDCD4 in turn can be suppressed by miR-21, and inhibition of miR-21 in RKO colon cancer cells reduced intravasation in the CAM assay (3).

The mechanism by which the uPA/uPAR system enhances intravasation may be multifactorial, given that plasmin can digest a wide range of proteins. One example of a downstream mediator for intravasation is CDCP1. A CDCP1-specific antibody that blocks plasmin cleavage of CDCP1, mAB 10-D7, was able to block intravasation of prostate cancer cells in the CAM assay, as did treatment with aprotinin (14). mAB 10-D7 was also able to inhibit protease-stimulated intravasation using an in vitro transendothelial migration assay. Cleaved CDCP1 forms a complex with β_1 -integrins, resulting in FAK activation and PI3K activation. Inhibition of FAK or PI3K activity resulted in reduced intravasation.

Other proteases that may contribute to intravasation include the matrix metalloproteinases (MMPs). However, the literature is mixed regarding the role of MMPs in intravasation. The MMP inhibitor marimastat strongly inhibited intravasation of HEp3 cells in the CAM assay (61), while the MMP inhibitor GM6001 inhibited fibrosarcoma but not prostate cancer intravasation (7, 30). Both papers report that uPA/uPAR is important. GM6001 did inhibit block transendothelial mi-

gration of breast cancer cells in an in vitro intravasation assay, however (99).

This mixed record is also reflected in studies of individual MMPs. In the CAM assay, suppression of MMP-1 expression in HEp3 cells resulted in reduced intravasation, possibly due to perturbed angiogenesis and endothelial permeability via regulation of endothelial PAR1 (52). Conversely, although GM6001 inhibited intravasation of fibrosarcoma in the CAM assay, MT1MMP suppression had no effect while MMP-1, MMP-2, or MMP-9 suppression was found to actually increase intravasation (30). In the fibrosarcoma case, one possibility is that tumor cell and host MMPs have contrasting contributions to intravasation in this system. Host MMPs may contribute to angiogenesis, with increased angiogenesis enhancing the likelihood of intravasation (8, 29, 74). Overexpression of membrane type MMPs in breast cancer cells can enhance intravasation, potentially through stimulating invasion or perturbing angiogenesis and generating larger, more permeable blood vessels (15, 82).

Antitumor treatments may induce increased intravasation in parallel with cell killing. In a mouse model using lung cancer cells, irradiation resulted in increased intravasation and MMP-9 expression. Suppression of MMP-9 inhibited the radiation-induced increase in intravasation (19). In wild-type p53 lung cancer cells, irradiation led to the induction of SULF2 which in turn upregulated IL6 expression, invasion, and intravasation (53).

Other Contributors

Other receptors that can contribute to intravasation include G protein-coupled receptors. CXCR1 and CXCR2 are upregulated by CYR61/CCN1 expression in gastric cancer cells, and neutralizing antibodies for CXCR1 and CXCR2 inhibit intravasation induced by CCN1 expression in the CAM assay (67). Expression of the SDF1/CXCL12 receptor CXCR4 in MTLn3 breast cancer cells could enhance intravasation in a mouse model, while CXCR7 could not (48), although CXCR7 enhanced primary tumor growth through angiogenesis. A role for Notch signaling in colon cancer cells has been shown, where loss of an inhibitor of Notch, Aes, resulted in increased invasion of tumor glands into blood vessels (109).

A number of other potential contributors to intravasation have been identified. Oxidative stress and reactive oxygen species generated by UV radiation were shown to enhance basal to apical transendothelial migration of melanoma cells using an in vitro assay (18). Tissue factor expression on osteosarcoma cells enhanced intravasation in the CAM assay (23).

Regulation of tumor cell adhesion interactions can also perturb intravasation. Increased cell-cell adhesion through ICAM1 in a T-cell leukemia model resulted in reduced intravasation (33). Increased osteopontin expression can enhance intravasation in the CAM assay (59). An antibody to the tetraspanin CD151 inhibited tumor cell motility and intravasation without affecting proliferation (143). $\alpha_2\beta_1$ -integrin expression inhibits intravasation of the MMTV-Neu mouse model of breast cancer (97). Conversely, β_3 -integrin expression was important for metastasis of MDA-MB-231 breast cancer cells (13).

Other Participating Cells

Tumor cell intravasation can be facilitated by other cells in the tumor stroma. Macrophages, fibroblasts, neutrophils, and platelets can all aid in migration across the endothelial barrier.

While macrophages normally play a role in tumor suppression by eliminating damaged and mutated cells, it has been shown that macrophages can be reprogrammed by tumor cells to support tumor growth and help in the metastatic spread of cancer (21, 95). One such relationship is the EGF/CSF1 paracrine interaction between breast cancer cells and macrophages. Tumor cells secrete CSF1 to recruit macrophages, and in turn macrophages secrete EGF in order to stimulate tumor cell invasion towards blood vessels (44). Once at the endothelial barrier, both the tumor cell and macrophage produce proteases at the site of tumor cell entry (130), showing a direct interaction between tumor cells and macrophages involved in hematogenous intravasation. This interaction is contact dependent as shown by Roh et al. (99), where contact between tumor cells and macrophages induces the formation of tumor cell invadopodia, allowing the cell to penetrate the endothelial tight junctions and migrate into the bloodstream. One population of tumor-associated macrophages, perivascular macrophages, is of particular interest. It has been shown that tumor cells tend to intravasate in areas enriched for these perivascular macrophages, which express VEGFA (46, 130). Quantifying the association of a Mena-expressing tumor cell with a perivascular macrophage and an endothelial cell, termed TMEM (tumor microenvironment of metastasis), may have prognostic value (98).

Macrophage-secreted TNFA may also play a role in mediating tumor cell intravasation. Studies by Zervantonakis et al. (139) show that treating an endothelial cell monolayer with TNFA from macrophages can increase endothelial layer permeability. This has been further supported in a recent study by Wang et al. (123) in zebrafish, showing that tumor-associated macrophages bind to tumor cells to aid in intravasation. In their findings, the authors show that macrophage TNFA and IL6 are important signaling molecules for this step (123).

Cancer-associated fibroblasts (CAFs) can interact with tumor cells and mediate migration through a CXCL12/CXCR4 paracrine loop. Much like the paracrine interaction between tumor cells and macrophages, this allows for migration through tissue and towards blood vessels (37, 75). Pena et al. (88) have found that STC1, a protein expressed by PDGF-activated fibroblasts, may be important for colorectal cancer cell intravasation. Tumors formed in mice with STC1-deficient CAFs showed decreased numbers of tumor cell emboli in the tumor vasculature (88).

Neutrophils play a role in assisting tumor angiogenesis and intravasation by being a source of MMP-9, a metalloproteinase that stimulates the production of new blood vessels within the tumor. In a study using aggressive fibrosarcoma and prostate carcinoma cells, it was found that the ability of these cells to cross the endothelial barrier was dependent on how well they were able to recruit MMP-9-positive neutrophils (8).

Platelets, like many of the other interacting cells in the tumor microenvironment, are a source of several growth factors that are important for cancer cell intravasation. Platelet-derived growth factor, PDGF, has been shown to increase tumor cell

proliferation, as well as activate other cells within the tumor stroma, such as fibroblasts. TGFB, a protein released by the alpha granules of platelets, can promote cancer cell intravasation as described above.

Circulating Tumor Cells

A number of studies have shown that the presence of circulating tumor cells (CTCs) correlates with disease progression and metastasis in different human cancers (20, 24, 27, 76, 78). For example, in a study of metastatic breast cancer, patients with 5 or more CTCs per 7.5 ml of whole blood had a significantly shorter median progression-free survival and shorter overall survival as compared with patients with less than 5 CTCs (24). Rapidly advancing technologies to measure CTCs are allowing many studies to utilize the measurement of CTCs in model systems to validate analyses performed in human cancers and provide novel insights into the mechanisms of intravasation.

In a study using the 4T1 breast cancer model, the gene expression patterns of CTCs were compared with primary tumor cells (65). There were significant increases in TWIST1 and SNAI1, consistent with increased EMT in intravasating cells and studies indicating that TWIST1 expression can enhance metastasis (134). In addition, there was a significant increase in the mitochondrial inducer, PGC-1, and intravasation correlated with PGC-1 expression in both animal studies and patient CTCs. In another study of CTCs during treatment, changes in gene expression were followed and EMT patterns were detected (136). However, in this study, direct comparisons with the tumors giving rise to the CTCs were not made, and thus it is unclear in this case whether the CTC signatures simply reflected the expression in the source tumor. Genomic signatures of CTCs were characterized in a study of 40 breast cancer patients (54). CTCs from the peripheral blood of patients were isolated using single-cell laser microdissection and analyzed for copy number aberrations (CNA) by whole genome amplification. A signature of recurrent copy number gains was identified containing genes and miRNAs which have known roles in motility, invasion, and intravasation. Examples that could contribute to enhanced intravasation as discussed above include PAI-1, CD151, ErbB2, and ANGPTL4. Furthermore, the analysis clustered the CTCs into two broad signatures: dormancy-related versus tumor-aggressiveness related. However, there are a number of potential complications in interpreting the results, including whether the CTCs are from metastases or the primary tumor, possible contamination with lymphoid cells, and how the CTCs have avoided being trapped in capillaries (93).

Aside from tumor expression of key proteins and miRNA, extracellular elements in the blood may also regulate CTCs. The role of platelets in CTC survival and colonization has been reviewed (111). Briefly, once in the circulation, CTCs activate and cause aggregation of platelets, leading to the formation of tumor cell-induced platelet aggregations (TCIPA). As aggregates, platelets enhance survival and extravasation of CTCs into new metastatic sites by facilitating the dissemination and embolization in the microvasculature as well as protection from immunological attacks.

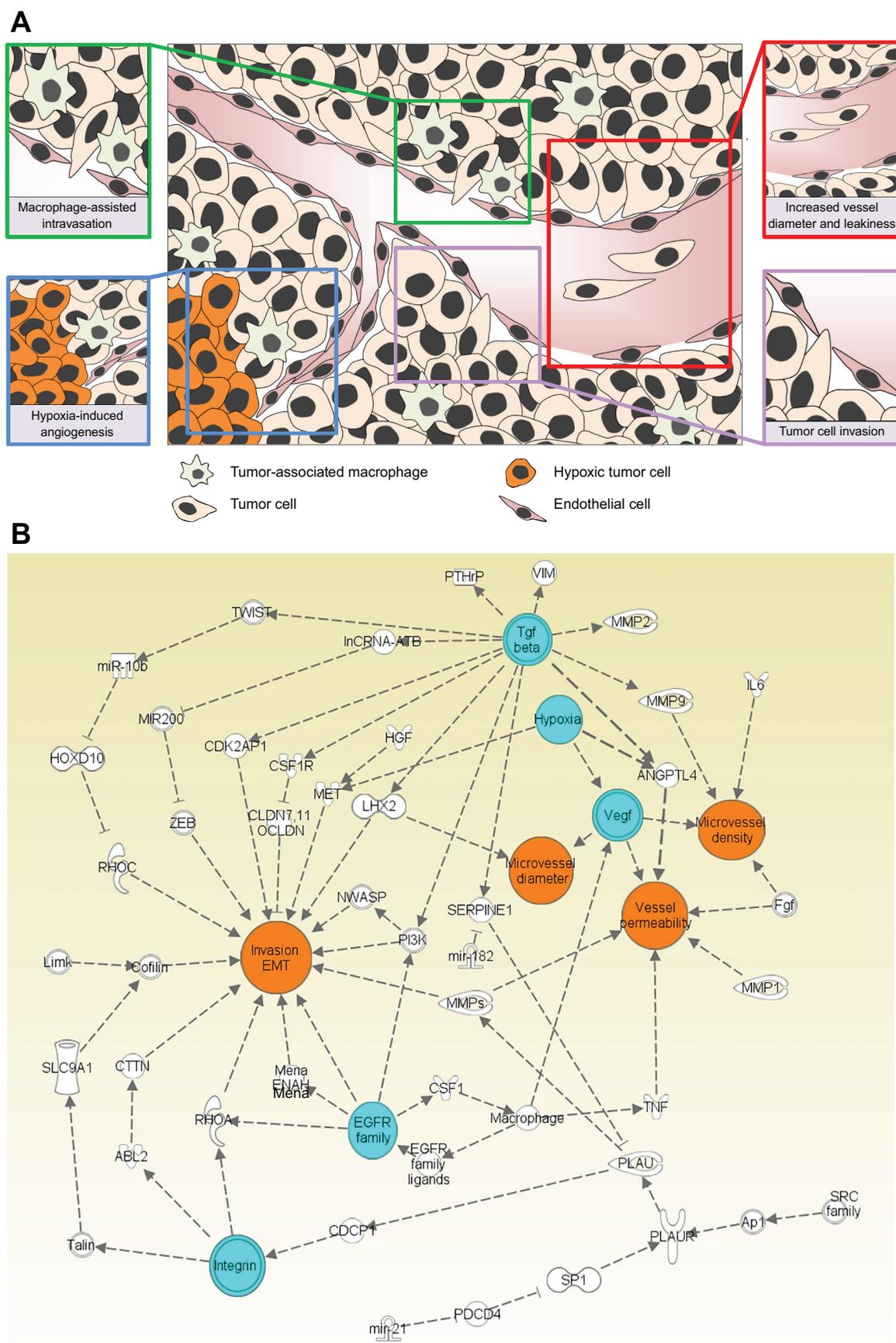


Fig. 2. Summary of intravasation mechanisms. **A**: diagram summarizing basic contributors to intravasation. **B**: interaction network of the major mechanisms described in this review. Major stimulators of intravasation are noted in blue and key properties regulating intravasation are indicated in orange.

Conclusions and Future Prospects

A number of molecular pathways for regulation of intravasation have been identified (Fig. 2). The development of sophisticated in vivo and in vitro assays for the analysis of intravasation has been important for our current advances and will enable more detailed understanding of the events that take place during intravasation. It is possible that different tumor types (or subtypes) will utilize different mechanisms of intravasation, and this will be important to clarify development of clinical applications. The expression patterns of CTCs may be helpful in confirming the pathways that are important for intravasation.

Possible clinical applications of our understanding of intravasation broadly fall into two general categories: prognosis and therapy. With respect to prognosis, measurements of histological grading, staging, and hormone receptor status are used by clinicians to determine the most appropriate treatments. However, these clinical criteria only roughly assess intravasation capability (and the resulting metastatic capability). Mechanism-based tests have the potential to provide additional prognostic value. For example, the observation that macrophages can enhance tumor cell intravasation capability through increases in the expression of Mena/ENAH has been used to develop a new test based on the tumor microenvironment of metastasis (TMEM). TMEM has been shown to independently predict the risk of metastasis (81, 91). The ability to more accurately identify which patients are unlikely to have metastases could significantly reduce the complications due to unnecessary chemotherapy and/or radiation therapy.

Development of new therapies based on anti-intravasation treatments is complex. If an anti-intravasation therapy does not affect tumor cell viability, then it may simply constrain the tumor cells to the primary tumor. This can potentially be useful for reducing tumor cell dissemination induced by other treatments (such as radiation as noted above) and may also enhance killing efficiency of the combined therapy. This may be more effective in treating tumors where complete removal of the primary tumor with adequate margins is often difficult (such as prostate or head and neck cancer). For tumors such as breast, in which the primary tumor often can be completely removed, anti-intravasation therapy may be useful to treat metastases once they recur. In this context, it will be important to determine whether the mechanism of intravasation from a metastasis is the same as intravasation from the primary tumor.

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AUTHOR CONTRIBUTIONS

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