Physical and Chemical Gradients in the Tumor Microenvironment Regulate Tumor Cell Invasion, Migration, and Metastasis

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Cancer metastasis requires the invasion of tumor cells into the stroma and the directed migration of tumor cells through the stroma toward the vasculature and lymphatics where they can disseminate and colonize secondary organs. Physical and biochemical gradients that form within the primary tumor tissue promote tumor cell invasion and drive persistent migration toward blood vessels and the lymphatics to facilitate tumor cell dissemination. These microenvironment cues include hypoxia and pH gradients, gradients of soluble cues that induce chemotaxis, and ions that facilitate galvanotaxis, as well as modifications to the concentration, organization, and stiffness of the extracellular matrix that produce haptotactic, alignotactic, and durotactic gradients. These gradients form through dynamic interactions between the tumor cells and the resident fibroblasts, adipocytes, nerves, endothelial cells, infiltrating immune cells, and mesenchymal stem cells. Malignant progression results from the integrated response of the tumor to these extrinsic physical and chemical cues. Here, we first describe how these physical and chemical gradients develop, and we discuss their role in tumor progression. We then review assays to study these gradients.

We conclude with a discussion of clinical strategies used to detect and inhibit these gradients in tumors and of new intervention opportunities. Clarifying the role of these gradients in tumor evolution offers a unique approach to target metastasis.

Tumors are highly heterogeneous and this feature compromises treatment efficacy and promotes tumor cell dissemination and metastasis to reduce patient survival. Tumor heterogeneity is driven in part by genetic alterations induced through genomic instability and maintained by the evolutionary selection of these genetically modified cells (Alizadeh et al. 2015). Epigenetic, transcriptional, and posttranslational modifications also contribute significantly to tumor cell diversity. Furthermore, cancer develops within a complex tissue microenvironment that itself fosters tumor heterogeneity through clonal selection as well as via epigenetic reprogramming and modifications of the tumor phenotype. The tumor microenvironment is composed of cellular constituents that include cells of the vasculature, infiltrating immune cells, and resident fibroblasts, adipocytes and nerves, and a noncellular component composed of diverse soluble factors and a highly variable and evolving insoluble extracellular matrix (ECM) (Joyce and Pollard 2009). The composition and organization of the tumor microenvironment vary significantly within and across tumors.

One key feature of the noncellular microenvironment is the existence of local chemical and physical gradients that exert profound effects on the tumor phenotype, including its predilection to disseminate. In this regard, metastasis is facilitated by efficient local tumor cell invasion that is fostered by ECM and chemokine/cytokine/growth factor gradients which cooperate to promote the directional migration of the tumor cells toward the vasculature and lymphatics and facilitates their extravasation from the primary tumor site into the circulation and their intravasation into the distal tissue. In this review, we discuss the role of directional migration in cancer progression and metastasis in the context of local microenvironmental gradients that develop in the primary and metastatic tumor tissue. We begin by reviewing the generation of endogenous gradients within both the primary and metastatic tumor tissues. We then summarize some of the techniques currently used to study directional migration in cancer and discuss putative signaling mechanisms regulating directional migration in response to external cues and the intrinsic state of the tumor cell. We have
chosen to focus exclusively on methods applied to the study of cancer progression and mechanisms driving directional migration of tumor cells. Finally, we discuss existing approaches available to interfere with directional migration that could prevent tumor metastasis and outline potential future avenues for therapeutic development.

**TUMOR HETEROGENEITY LEADS TO ENDOGENOUS GRADIENT GENERATION IN TUMORS**

**Local Gradients of Secreted Factors**

Chemokines and growth factors (GFs) are key drivers of tumor cell invasion and facilitate tumor cell extravasation and intravasation to promote metastasis. Tumor cells secrete chemoattractants for macrophages, neutrophils, lymphocytes, fibroblasts, and mesenchymal stem cells that once recruited secrete chemokines and GFs that stimulate tumor cell migration (Roussos et al. 2011). The specific, directed migration of stromal cells into the tumor and the reciprocal persistent migration of the tumor cells into and through the stroma are reinforced by the restricted expression of specific receptors for each of the plethora of secreted factors. For example, tumor-associated macrophages (TAMs) release growth factors, including epidermal growth factor (EGF), that activate the EGFR receptor expressed on the breast tumor cells to stimulate and recruit TAMs through TAM-specific expression of the CSF-1 receptor. This vicious feed-forward paracrine loop between the invading tumor cells and infiltrating macrophages ensures the persistent migration of the tumor cells toward the vasculature that is key for tumor cell dissemination and metastasis. The chemokine CXCL12, produced by pericytes and tumor cells, can also drive in breast tumor cell chemotaxis in vivo (Müller et al. 2001). Although the molecular details remain to be elaborated, several in vitro studies have attested to the existence of similar local reciprocal gradients of GFs such as EGF ligands, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), and CSF-1, in a variety of tumor types, investigating how this interplay stimulates stromal cell infiltration to potentiate tumor cell migration and dissemination (Roussos et al. 2011). Interestingly, tumor cells themselves can also create local gradients to facilitate their directed migration as has been shown through their matrix metalloproteinase-mediated degradation of the ECM or as was recently illustrated for melanoma tumor cells that create their own lipid chemoattractant lysophosphatidic acid gradient to foster their migration (Muñonen-Martin et al. 2014; Tweedy et al. 2016). Furthermore, soluble gradients have been implicated in tissue tropism within the context of metastasis and distal organ colonization. Indeed, chemokines secreted by cancer-associated fibroblasts (CAF) have been associated with local stromal cells secreting a chemokine whose receptor is located on the tumor cell. For example, CAF-secreted CXCL12/SDF-1 drives metastasis of CXCR4-expressing breast cancer cells to the lymph nodes and lung (Müller et al. 2001), with CCL27 driving CCR10-dependent skin metastasis of melanoma and CCL21/CCL19 activating CCR7 to promote lymph node metastasis (Ben-Baruch 2008) (Figs. 1 and 2).

**Changes in ECM Abundance and Organization**

The ECM is a complex scaffold of proteins that provide structural support to the surrounding tissue. Increased deposition and accelerated post-translational modifications including cross-linking of the ECM are characteristic features of the tumor desmoplastic response that is associated with poor patient prognosis (Walker 2001). Recently, proteomics of the decellularized, soluble ECM fraction of the tumor tissue identified core matrix-some proteins and found that this signature contains as many as 274 proteins composed of glycoproteins, collagens, and proteoglycans (Naba et al. 2012). Intriguingly, using this approach and relying on analysis that uses species-specific peptides, it was revealed that many of the tumor ECM proteins including a number of abundant laminins (LAMA3, B3, C2) and Sned1 are secreted predominantly by the tumor cells, whereas other ECM components such as tenascin C (TNC) and vitronectin are secreted exclusively by resident fibroblasts and infiltrating stromal cells (Naba et al. 2014). Complementing these studies, advanced proteomic approaches have been able to profile the insoluble ECM proteins within the tumor, revealing distinct insoluble protein profiles within a tumor type that not only reflect the tumor stage but also the tumor genotype (Goddard et al. 2016; Laklai et al. 2016). Data revealed that the most aggressive pancreatic tumors that harbor SMAD4 mutations and that are associated with the shortest patient survival deposited quantities of collagen 12, TNC, and fibronectin (FN) nests surrounding the invading ductal lesions (Laklai et al. 2016). Overall, multiple proteomic studies have permitted researchers to conclude that the ECM is temporally modulated, spatially distinct, and influenced by the tumor genotype, and that its phenotype is dictated by both the tumor cells themselves as well as the resident and infiltrating stromal cells (Naba et al. 2014, 2016). For instance, FN, which is typically elevated in tumors, is highly heterogeneous with the highest levels found at the tissue periphery, near the vasculature and associated with thick collagen bundles (Astrof and Hynes 2009; Zhang et al. 2014; Miroshnikova et al. (in review). Peripheral solid tumors are typically fibrotic and characterized by elevated reorganized collagens that are often lysyl oxidase cross-linked into bundles upon which tumor cells have been seen migrating (Wang et al. 2004; Wyckoff et al. 2006; Erler and Weaver 2009). Using two-photon imaging has made it possible to characterize tumor collagen organization, and using structured illumination polarized imaging informs on the degree of alignment.
These two approaches have revealed that the thick, highly aligned, fibrillar collagens typically observed in a primary tumor are the result of the dramatic reorganization of the relaxed, coiled short fibers found in a normal tissue (Levental et al. 2009). Provocatively, the organization of the tumor-associated fibrillar collagens is highly heterogeneous with prominent oriented fibers frequently localized to the invasive front, associated with invading blood vessels, and around the tumor periphery (Provenzano et al. 2006; Levental et al. 2009; Lopez et al. 2011; Acerbi et al. 2015). The clinical relevance of collagen reorganization at the primary tumor was emphasized by a retrospective study in which the presence of perpendicular collagen bundles in invasive human breast tumors was found to associate with poor patient outcome (Egeblad et al. 2010; Conklin and Keely 2012). Intriguingly, recent work revealed that several matricellular proteins, such as FN and TNC, can also accumulate in the premetastatic niche and may serve to attract and subsequently enhance tumor cell growth and survival, particularly because they are deposited as a highly endogenous gradient in the niche (Kaplan et al. 2005; Costa-Silva et al. 2015). Evidence to support this possibility was provided by experimental data showing that TNC is a major gene that is significantly up-regulated in breast tumor cells that preferentially colonize the lung (Minn et al. 2005).

Stiffness Gradients

Tumors are mechanically corrupted tissues whose physical attributes typically include high tumor solid stress that reflects the tumor genotype and is promoted by the expanding tumor cell mass, the elevated interstitial pressure, and the resistance exerted by the surrounding stiffened, fibrotic ECM (Padera et al. 2004; Paszek et al. 2005; Willipinski-Stapelfeldt et al. 2005). ECM stiffening reflects an increase in protein abundance; particularly of fibrillar proteins such as collagens I and III, as well as their reorganization and posttranslation modifications that include lysyl oxidase–mediated cross-linking (Erler and Weaver 2009; Levental et al. 2009; Egeblad et al. 2010; Cox et al. 2016). Although imaging elastography and unconfined compression analysis have consistently revealed an incremental increase in tissue stiffness as a function of tumor stage, grade atomic force microscopy (AFM) indentation analysis has also emphasized the heterogeneity of the ECM stiffness within a tumor (Evans et al. 2012; Plodinec et al. 2012; Yi et al. 2013; Fenner et al. 2014). For instance, AFM indentation revealed that the invasive front of human tumors is by far the stiffest (Acerbi et al. 2015) and determined that the vasculature within the core of the tumor is softer than the vasculature at the periphery (Lopez et al. 2011); possibly reflecting...
differences in pericyte coverage (Ribeiro and Okamoto 2015). These distinct regions of ECM stiffness gradients that can foster tumor cell migration through a process termed durotaxis (Lo et al. 2000; Isenberg et al. 2009; Plotnikov et al. 2012). Interestingly, two common organs of secondary metastasis are the liver and the lung (Wynn 2008; Steeg 2016), both of which suffer from age, disease, and obesity-mediated chronic fibrosis (Zeisberg and Kalluri 2013). Chronic liver and lung fibrosis are characterized by tissue stiffening through elevated levels of fibrillar-type collagens, TNC and FN, and more proteoglycan and lysyl oxidase-mediated cross-linking, which would comprise a strong stiffness differential gradient toward which disseminated tumor cells would be attracted (Levental et al. 2009; Chang et al. 2017).

Establishment of Hypoxia

Tumors are hypoxic (Wilson and Hay 2011) as a result of insufficient or dysfunctional vasculature that is often leaky, heterogeneous, and tortuous. This irregular branching and abnormal shunting lead to heterogeneous blood flow (Carmeliet and Jain 2011), which in turn creates endogenous hypoxia gradients within the tumor that can include extremely low pO2 levels in some areas of the tumor (Li et al. 2007). The tumors adapt to the reduced oxygen by adjusting their intrinsic metabolism, which creates a layered metabolite gradient within the tissue. Thus, vascular heterogeneity creates gradients of extracellular oxygen levels or pO2, which alter the intravascular pO2 and the metabolic consumption rate of the tumor and stromal cells within the tissue. The combination of these events creates pO2 and metabolite gradients within the tissue that can have a profound effect on tumor cell behavior.

Inversion of Normal pH Gradient

In differentiated epithelial cells, the intracellular pH (pHi, 7.2) is lower than the extracellular pH (pHe, 7.4). However, in a tumor tissue the pH can change dramatically depending on the pO2 and metabolite availability, resulting in a reversal of the normal pH gradient (Webb et al. 2011), with cancer cells developing a higher pHi of 7.4 and much lower pHe of 6.5–7.1 (Stüwe et al. 2007; Gallagher et al. 2008; Hashim et al. 2011). Changes in pH in tumor cells can occur as a consequence of up-regulation of expression and/or activity of ion pumps and transporters, such as H⁺ ATPases (Martinez-Zagulian et al. 1993), Na⁺–H⁺ exchanger NHE1 of the SLC9A family (McLean et al. 2000), and monocarboxylate–H⁺ efflux cotransporters MCT1 and MCT4 or the SLC16A family (Pinheiro et al. 2008; Chiche et al. 2012) that drive H⁺ efflux, leading to a high pHe. Furthermore, high activity of carbonic anhydrases CAIX and CAXII driven by hypoxia can accelerate the hydration of extracellular CO₂ to HCO₃⁻ and H⁺ (Pastorekova et al. 2006; Chiche et al. 2009; Swietach et al. 2009). Finally, reduced blood flow and subsequent oxygen depletion at the early “carcinoma in situ” stage will drive tumor cells to undergo a metabolic switch toward a glycolytic phenotype (Gatenby and Gillies 2004). The increased flux of carbons resulting from glycolysis leads to an acidification of the extracellular pH of the tumors that is particularly pronounced in luminal structures (Barathova et al. 2008). Importantly, similar to pO2 and metabolite gradients, tumors can also develop pH gradients that can influence their behavior and additionally contribute to tumor heterogeneity.

Alterations in Electrical Fields

Electrical signaling plays a fundamental role in cell and tissue homeostasis. The membrane potential of a cell is maintained by the unequal distribution of ions across the plasma membrane, which creates voltage differences between the cytoplasm and the extracellular environment (Yang and Brackenbury 2013). In epithelial tissues, naturally occurring electrical fields (EFs) are maintained by the spatial organization of ion pumps, with the apical domain of the cell being especially enriched in Na⁺ channels and Cl⁻ transporters, and the basolateral domain containing Na⁺–K⁺ ATPases (McCaug et al. 2009). The generation of ionic gradients leads to extracellular ionic current flow and the establishment of endogenous voltage gradients. Normal epithelial cells are highly polarized; however, the membrane potential of tumor cells is often depolarized, and this difference results in a drop in transepithelial potential difference (James et al. 1956; Faupel et al. 1997). Depolarized tumor cells display in-
creased intracellular Na\(^+\) levels, stable K\(^+\) levels, and Cl\(^-\) and Ca\(^{2+}\) influx (Yang and Brackenbury 2013). Tumor depolarization results in an extracellular voltage gradient between normal and tumor cells (Cuzick et al. 1998) that can vary widely within one tumor and differs between tumor types. For example, across the luminal wall of rat prostate glands, a steady direct current electrical field of 500 mV/mm exists, with a transepithelial electrical potential difference of \(-10\) mV, the lumen being negatively charged (Sztatkowski et al. 2000; Djamgoz et al. 2001). In contrast, in the mammary duct, the transepithelial electrical potential is \(+30\) mV, with duct lumen being positively charged (Pu et al. 2007).

### TECHNIQUES TO STUDY DIRECTIONAL MIGRATION

The transwell or Boyden chamber assay (Boyden 1962) is the original assay used to study directional migration, in which cells are plated on a porous filter membrane in between two cell culture chambers. By layering a soluble or ECM cue in only one of the two chambers, it is possible to study directional migration through the filter toward a cue of interest. Although this assay has unequivocally enhanced our understanding of directional migration, the approach is fraught with many limitations that include most importantly an inability to control the local environment and to generate gradient slopes. Furthermore, the technique does not easily lend itself to live imaging. In recent years, the convergence of bioengineering and cancer research has helped facilitate the development of more accurate and reliable methods to study directional migration, both in vitro and in vivo (see Fig. 3).

#### Micropipette/Micropatterning Assays for Local Two-Dimensional Gradients In Vitro

The first directional migration assay that was developed to generate local quantifiable gradients involved the use of a micromanipulator-controlled micropipette, loaded with a given concentration of a soluble cue that was slowly released in proximity to a two-dimensional (2D)-plated cell. In this assay, micropipettes are placed within 1–2 cell diameters of each cell, and a pump is used to control the flow of the chemoattractant out of the pipette and, in turn, the gradient (Mouneimne et al. 2004; Soon et al. 2005). This type of assay has been used successfully to examine carcinoma cell responses to soluble GF gradients (Carmona et al. 2016). However, this method is low-throughput, technically tedious, and requires expensive equipment. Moreover, the gradients generated by the micropipette approach are transient, making it difficult to quantify chronic cellular changes, and lend themselves only to the study of the effect of soluble cues on cell behavior. To address this issue, 2D micropatterning has been developed to study durotactic gradients. Although these 2D durotactic gradients have enjoyed varying degrees of success, they are notoriously technically challenging and thus not easily adapted to most cancer cell biology laboratories. To facilitate these studies, new durotactic gradient assays have been developed using mechanically tunable 2D polymer hydrogels conjugated with different ECM ligands (Roca-Cusachs et al. 2013). In these assays, stiffness gradients are generated by vary-
ing the cross-linking density of polyacrylamide hydrogels (Kuo et al. 2012), controlled by the amount of the cross-linker or light, for photoinitiated polymerization of gels (Sunyer et al. 2012). Alternately, a three-dimensional (3D) durotaxis gradient can be generated using a novel 3D collagen/FN/basement membrane hydrogel bioreactor that rapidly induces a 3D stiffness gradient by varying the angle of uniaxial stretch (Cassereau et al. 2015).

**New Advances in Microfluidics**

Advances in microfluidic technology have paved the way for its application to the study of directional migration. These tailor-made microfluidic devices permit precise spatiotemporal control over the microenvironment the cells are in and the gradients toward which cells will migrate. At present the vast majority of these microfluidic devices use polydimethyl-siloxane (PDMS), which facilitates the generation of microfabricated patterns and structures. Microfluidic devices are easily adopted in a cell biology platform and have the added benefit that they are reasonably cheap to generate. They also use small volumes of reagents, can be easily manipulated, and lend themselves to high-resolution time-lapse imaging (Bersini et al. 2014). To this end, microfluidic devices have been used to study the impact of soluble gradients of growth factors on cancer cell migration with good success (Li and Lin 2011). Some of the newer generation microfluidic devices incorporate pumps that provide a continuous flow of the soluble gradient and permit intermittent fluid analysis (Wu et al. 2012), whereas others generate long-term stable gradients and have revealed that subtle differences in gradients can exert significant biological effects (Saraneni et al. 2012). Stable ECM haptotactic gradients have also been developed using microfluidics and used to study 2D and 3D responses to multiple ECM cues such as FN, laminin, and vitronectin (Chan et al. 2014; Oudin et al. 2016a). Microfluidic channel width can also be used to directly modulate collagen alignment. In these iterations, a flow-based 3D microchannel assay that plated collagen in wide channels (3 mm) produced a random matrix, whereas when the collagen was added to a narrow channel (1 mm), it produced an aligned collagen matrix (Riching et al. 2014). Finally, microfluidics have significantly improved the study of galvanotaxis, which requires stable, integrated, tightly sealed cell culture chambers to maintain the provision of multiple stable electric fields (Huang et al. 2009; Li and Lin 2011).

**Visualizing and Measuring Directional Migration In Vivo**

Generating any sustained gradient—whether it is a soluble factor, a mechanical constraint, or a biochemical cue—within an endogenous tumor microenvironment in vivo is extremely challenging. One assay developed to study tumor cell migration responses to local gradients in vivo is the needle collection or in vivo invasion assay. In these assays, stainless steel needles containing an attractant are placed within an endogenous tumor into an anesthetized mouse that has developed a genetically engineered or a xenografted tumor (Hernandez et al. 2009). Intravital imaging studies in mice containing needles with EGF have shown that cells enter the needle by active migration only (Wyckoff et al. 2004). Since its inception this assay has been used to study both chemotactic and haptotactic responses (Oudin et al. 2016a). More recently, a new microscale device was developed which incorporates multiple reservoirs in the delivery system to permit the time-resolved delivery of several compounds. This new device permits the slow diffusion of drugs over time (Jonas et al. 2015). These devices can be placed on the edges of tumors and, coupled with intravital imaging, can permit real-time imaging of the tumor cells response to large sustained local gradients in vivo (Oudin et al. 2016a). Other implantable devices have been developed, such as the iNanovid (Williams et al. 2016b), to visualize cell responses in vivo directly, and these approaches are likely to become far more powerful in the near future. Overall, in vivo directional migration studies are technically difficult, require specific noncommercially available equipment, and are expensive. Nevertheless, they serve to validate the importance of microenvironmental gradients as a key regulator of the tumor phenotype.

**MECHANISMS OF DIRECTIONAL MIGRATION**

Directional migration favors the invasion of tumor cells into the parenchyma and their metastatic dissemination and colonization. The basic mechanisms driving cell migration, as well as the different modes a cell can use to migrate, have been described in detail elsewhere (Friedl and Wolf 2003; Petrie et al. 2009). Directional migration occurs through a series of defined steps that include cell polarization via asymmetric protrusion formation, focal adhesion formation and adhesion, and cell contraction leading to rear end detachment. Here, we focus specifically on mechanisms that have been shown to be involved in the directed migration of cancer cells and that have been implicated in cancer progression. The pathways described below have been summarized in Figure 4.

**Chemotaxis**

Numerous soluble cues drive chemotaxis by stimulating actin polymerization through RhoGTPases through the cells’ cognate receptor tyrosine kinase (RTK) receptors or Gpi protein-coupled receptors (GPCRs) depending on the ligand (for review, see Roussos et al. 2011). In breast cancer cells, multiple GFs such as EGF, heparin-binding (HB)-EGF, TGF-α, insulin-like growth factor (IGF), and hepatocyte growth factor (HGF), but not PDGF and heregulin, induce strong protrusion responses that are predicted to drive 3D invasion in vitro (Meyer et al. 2012). Interestingly, the in vivo invasion assay revealed that EGF, TGF-α, and CSF-1, but not heregulin, VEGFα, fibroblast growth factor 1 (FGF-1), and PDGF, can induce the directed invasion of PyMT tumor cells in...
mammary tumors formed in polyoma middle T (PyMT)-mouse mammary tumor virus (MMTV) mice and identified a set of core signaling pathways including those that influence Rho/ROCK activity, the capping protein cofilin, and the Arp2/3 pathway (Wang et al. 2004; Wyckoff et al. 2004; Patsialou et al. 2012). These studies also identified the actin regulatory protein Mena, a member of the Ena/VASP family whose alternately spliced isoform MenaINV has been strongly implicated in breast metastasis as well as poor patient prognosis (Goswami et al. 2009; Roussos et al. 2010; Gertler and Condeelis 2011; Oudin et al. 2016a). Importantly, MenaINV deregulates the phoshatase PTP1B to enhance RTK phosphorylation and potentiate actin polymerization via the phospholipase Cγ (PLCγ)–cofilin pathway greatly potentiating the tumor cells’ response to chemotactic GFs such as EGF, IGF, and HGF (Philippar et al. 2008; Hughes et al. 2015). Chemotaxis downstream from GPCRs also contributes to tumor cell migration. For instance, GIV/Girdin, a non-receptor guanine nucleotide exchange factor (GEF) that triggers trimeric G-protein activation, binds to RTKs to promote metastasis (Garcia-Marcos et al. 2015), likely by fostering chemotaxis in response to insulin through enhanced Akt signaling. Similarly, chemokines such as CXCL12 (or SDF-1) can also drive chemotaxis via their cognate chemokine receptors.

**Haptotaxis**

The characteristic increase and heterogeneity in the distribution of the newly deposited and remodeled ECM protein in the tumor is consistent with the notion that haptotaxis contributes to the heterogeneity of the tumor phenotype. Haptotaxis is defined as the directed migration of cells on gradients of substrate-bound cues, in which cells are migrating on or within an ECM (Wu et al. 2012). In this respect, tumor haptotaxis toward gradients of the glycoprotein FN has been well studied. Studies using models of triple-negative breast cancer revealed that the actin binding protein Mena promotes FN haptotaxis through its ability to directly interact with the FN receptor integrin α5β1 (Oudin et al. 2016a). In this work, levels of the invasive isoform of Mena, MenaINV, whose expression associates with a poor breast cancer patient outcome, drove tumor cell haptotaxis toward high concentrations of FN by increasing focal adhesion kinase (FAK)-dependent outside-in signaling by enhancing inside-out signaling through ECM remodeling. The concept was validated in vivo using an implantable device to create FN gradients and intravital imaging to monitor and quantify effects on tumor cell migration. Importantly, these studies showed not only that FN can function as a bona fide directional cue, but also that a haptotaxis phenotype fosters tumor metastasis. Interestingly, in metastatic melanoma, loss of the tumor suppressor LKB1 and its downstream target, the MARK family kinases, compromises FN haptotaxis (Chan et al. 2014). Moreover, inserting a P29S mutation into the proinvasion, metastatic Rac1 molecule also abrogates fibroblast haptotaxis in vitro (King et al. 2016). Therefore, these data suggest context-specific effects of FN haptotaxis during tumor progression.

In addition to the haptotactic behavior of FN, Boyden chamber experiments have attested to the haptotactic potential of thrombospondin for human melanoma (Taraboletti et al. 1987). Given that thrombospondin is a glycoprotein localized at basement membrane and vessel
walls, this raises the possibility that circulating melanoma cells could feasibly encounter gradients of thrombospondin that could facilitate their invasavation into secondary organs to form metastatic lesions. Similarly, vitronectin, an ECM component involved in melanoma metastasis and present in serum, can act as haptotactic cue for A2058 melanoma cells by ligating αvβ3 integrin to promote the tyrosine phosphorylation of paxillin (Aznavoorian et al. 1996). Collagen, one of the most abundant ECM components in solid tumors, can also drive haptotaxis. Indeed, pancreatic tumor cells can drive haptotaxis in response to collagen by binding αvβ1 integrin to enhance FAK activity (Lu et al. 2014). GIV/Girdin, which promotes metastasis, is a nonreceptor GEF that triggers trimeric G-protein activation that binds to RTKs (Garcia-Marcos et al. 2015) and also activates integrins to promote haptotaxis toward collagen (Leyme et al. 2015).

**Durotaxis**

Cells preferentially migrate toward and remain associated with a stiffer ECM through a process termed durotaxis (Lo et al. 2000). Over the past several years, the original observations defining durotaxis have been elaborated upon using 2D and 3D natural and synthetic biomaterials with ECM stiffness gradients and have illustrated how cells persistently migrate up a stiffness gradient (Isenberg et al. 2009; Plotnikov et al. 2012; Cassereau et al. 2015). The importance of durotaxis to malignancy has also been shown by a series of in vitro studies using 3D hydrogels with calibrated stiffness and in vivo experiments in transgenic mouse models in which ECM rigidity was reduced using cross-linking inhibitors or enhanced through ECM cross-linking (for review, see Kai et al. 2016). These studies showed that a stiffened ECM promotes breast and squamous cell invasion by promoting integrin focal adhesions to enhance growth factor and GPCR signaling that activates RhoGTPases, Wnt, mitogen-activated protein (MAP), and phosphoinositide 3-kinases (PI3Ks) (Paszek et al. 2005; Levental et al. 2009). The relevance of ECM stiffness to breast tumor metastasis was further demonstrated by transgenic mouse studies in which collagen cross-linking and ECM stiffness were manipulated. These studies identified TGF-β and micro-RNAs that target tumor suppressors like the PI3K repressor phosphatase and tensin homolog (PTEN) as critical molecular modulators of the durotactic phenotype (Pickup et al. 2013; Mouw et al. 2014). Recently, a role for ECM rigidity in promoting pancreatic tumor progression and glioblastoma aggression that expand upon in vitro studies was demonstrated (Ulrich et al. 2009; Laklai et al. 2016; Miroshnikova et al. 2016). Building upon these observations data generated using MDA-MB-231 mesenchymal breast cancer cells defined an integrin-associated myosin-dependent molecular mechanism that regulates cellular rigidity sensing downstream from RTK signaling that is corrupted in tumors through AXL and ROR2 kinase perturbations (Lo et al. 2000; Hoffman et al. 2011; Plotnikov et al. 2012; Raab et al. 2012; Yang et al. 2016).

**Alignotaxis**

The reorganization of collagen from curly, coiled fibers to straight aligned fibers promotes the directed, rapid, and persistent migration of tumor cells, which we describe as alignotaxis. Alignotaxis was illustrated by studies conducted using the metastatic MDA-MB-231 breast cancer cells (Provenzano et al. 2008). Furthermore, aligned collagen fibers that are assembled through coordinated tumor colony interactions foster the rapid and directed migration of tumor cells (Shi et al. 2013). Consistently, intravital imaging studies in breast tumor models revealed that tumor cells located in close proximity to collagen fibers migrate faster than those located within the core of the tumor mass (Wang et al. 2002). Using fluorescently tagged myosin light chain (MLC), one study showed that MLC is recruited to the protrusions of migrating cells, with second-harmonic generation (SHG) imaging showing concomitant deformation in collagen fibers at the site of the protrusion (Wyckoff et al. 2006). Computational modeling followed by experimental validation showed that migratory persistence is enhanced by collagen fiber alignment because it limits the number of protrusions and restricts their formation to the direction of the alignment (Riching et al. 2014). Additional studies revealed that collagen fiber alignment can predict cell motility, relative to the pore size and density (Fraley et al. 2015), and that an increase in protrusion frequency, persistence, and lengthening along the direction of the fibers depends on FAK and Rac1 signaling (Carey et al. 2016). Indeed, preventing collagen alignment by inhibiting the activity of the collagen cross-linker LOXL2 (Grossman et al. 2016), or through knockdown of the proteoglycan syndecan1 (Yang et al. 2011), but not by inhibiting protease activity (matrix metalloproteinases [MMPs] and serine and cysteine proteases), reduced the directional migration of tumor cells in vitro and in vivo (Wyckoff et al. 2006; Levental et al. 2009; Rubashkin et al. 2014). Thus compelling in vitro and in vivo data attest to the importance of alignotaxis as a regulator of the local invasion of tumor cells.

**Galvanotaxis**

Galvanotaxis (or electrotaxis) describes the directional movement driven by different EFs. Multiple studies have illustrated a correlation between the metastatic potential of a tumor cell and its sensitivity to galvanotaxis (Djamgoz et al. 2001), including the galvanotaxis-mediated directional migration of prostate cancer cells (Djamgoz et al. 2001), breast cancer (Fraser et al. 2005), lung adenocarcinoma (Sun et al. 2012), and glioblastoma cells (Huang et al. 2016). Interestingly, although prostate, lung, and glioblastoma multiforme (GBM) tumor cells show cathodal galvanotaxis, the breast tumor cells migrate toward the anode, suggesting that different tumor microenvironments might contribute to EF-driven invasion. Although the primary stimulus differs, galvanotaxis likely exploits many of the same cytoskeletal pathways that mediate chemotaxis, haptotaxis, and durotaxis.
(Mycielska and Djamgoz 2004). For instance, EFs increase intracellular Ca\textsuperscript{2+} to induce actin polymerization and actomyosin contractility, which are key for the formation of localized cellular protrusions that permit directional migration (Mycielska and Djamgoz 2004). More recently, voltage-gated Na\textsuperscript{+} channels (VGSCs) were shown to control the galvanotaxis behavior of tumor cells, and their levels correlated with the tumor cells metastatic potential (Djamgoz et al. 2001; Fraser et al. 2005). More recently, A549 lung carcinoma galvanotaxing toward a cathode polarized their EGFRs and assembled filam- entous actin cables in the direction of the cathode. These galvanotaxing cells also showed oriented ERK and Akt signaling that lined up with the EF and apparently was critical for directional migration (Yan et al. 2009). Consis- tently, one study showed that the galvanotaxis of brain tumor initiating cells from three distinct GBM subtypes also depended upon the oriented activity of PI3K and ERK signaling (Huang et al. 2016). Although these findings suggest galvanotaxis can promote the directed migration of tumor cells, data clearly suggest that the nature of the behavior including the anodal versus the cathodal effect is context-dependent and likely cell type–dependent.

pH

The tumor-associated reversal of the normal pH gradient can profoundly influence intracellular signaling and modify the extracellular microenvironment to influence cell migration. Thus, the abnormally elevated intracellular pH\textsubscript{i} in the tumor cell can activate several intracellular signaling pathways linked to integrins, ion channel activity, and actin polymerization that will promote cell proliferation and migration. For instance, several guanine nucleotide exchange factors, such as DBS, that stimulate CDC42 become activated when the pH rises (Grillo-Hill et al. 2015). Other molecules that bind to actin and pro- mote polymerization, such as cofilin (Frantz et al. 2008), profilin (McLachlan et al. 2007), and talin (Srivastava et al. 2008), also have increased activity at higher pH. The extrusion of H\textsuperscript{+} from tumor cells is accompanied by an influx of water and subsequent osmotic swelling and opening of aquaporin channels (Saadoun et al. 2005), effects which contribute to driving cell migration. Consistently, the low pH\textsubscript{e} induced in tumor cells can significantly alter the ECM to create a prometastatic environment (Webb et al. 2011). Indeed, the lower pH\textsubscript{e} in the tumor microenvironment can activate multiple proteases such as MMP3 and MMP9 (Johnson et al. 2000), which result in ECM remodeling to foster the creation of migratory tracks and the release of ECM-bound soluble factors that stimulate cell motility.

### STRATEGIES TO TARGET DIRECTIONAL MIGRATION AND PREVENT METASTASIS

#### Exploiting Directional Migration to Predict Metastasis

The ability to predict which patients’ tumor has a high probability of metastasis would provide critical clinical insight to guide treatment and would likely influence the patient outcome. In this regard, a number of commercially available tests have recently been developed to estimate metastatic risk in cancer patients. One such test is the MammaPrint DX, which is a 70-gene signature that can identify breast cancer patients who are most likely to develop metastasis within 5 years of diagnosis with rea- sonable accuracy (van’t Veer et al. 2002; van de Vijver et al. 2002). Similarly, the 15-gene signature DecisionDX-UM test is able to accurately predict which patients with uveal melanoma are most likely to metastasize and has been tested in large multicenter trials with excellent results (Harbour 2014). Exploiting the concept of tumor gradients as key regulators of tumor migration and metastasis is the derivation of a new metastasis biomarker that quantifies the number of tumor microenvironment of metastasis (TMEM) sites within a tumor. TMEMs are a tri- partite structure composed of a macrophage, a Mena- expressing tumor cell, and an endothelial cell that result from and reflect chemotactic gradients within the tumor (Robinson et al. 2009). Recent clinical work suggests that as the number of TMEMs within an ER\textsuperscript{+}/PR\textsuperscript{+} breast tumor increases, metastasis increases; hence, patient prognosis decreases. The success of these new approaches to predict patient prognosis emphasizes the potential utility of developing directional migration biomarkers to predict metastatic disease (Rohan et al. 2014).

#### Blocking Cell Surface Receptors and Signaling to Impede Directional Migration

Therapies targeting the main chemotactic signaling pathways such as GFs/RTK and chemokine/GPCR have been approved for patient administration, with many more in clinical trials. Although most of these are aimed at inhibiting tumor growth and angiogenesis, they have the potential to impact metastasis-relevant processes. Yet, despite the fact that a role of EGF chemotaxis has been well established in breast cancer metastasis in mouse models, EGFR inhibitors have had mixed clinical efficacy (Crown et al. 2012). Small-molecule tyrosine kinase inhibitors (TKIs) such as gefitinib or erlotinib have failed to show any significant improvement in the triple-negative breast cancer (TNBC) patient outcome over standard-of-care chemotherapy (Baselga et al. 2005). The monoclonal EGFR antibody cetuximab showed only a modest increase in the overall response rate and progression-free survival relative to cisplatin (Baselga et al. 2013). Inhibitors targeting other surface RTKs that can modulate directional migration are currently in clinical trials for breast cancer, such as Met, IGF1R, FGFR, and ERBB3, and await verification of their utility (Jin and Mu 2015). Many molecular mechanisms are proposed to account for the failure of these promising RTK targets, including the expansion of genetically mutated tumor cells and reliance on parallel survival pathways.

Integrins are the main cell surface receptors that allow cells to sense, respond to, and reorganize their tumor mi-
microenvironment and cells use integrins for haptotaxis, chemotaxis, and durotaxis. However, although in vitro and preclinical studies have shown promising results (Schaffer et al. 2013), many of the integrin targeting clinical trials have ultimately failed (Desgrozeller and Cheresh 2010). The high-affinity anti-α5β1 antibody volociximab stabilized disease in metastatic renal cell carcinoma (Conti et al. 2013); however, another monoclonal α5β1 integrin antibody PF-04605412 had no effect on a variety of solid tumors and has consequently been discontinued (Mateo et al. 2014). A phase 3 trial in KRas wild-type metastatic colorectal cancer found no improvement with abituzumab, an anti-αv integrin antibody, relative to standard of care (Élez et al. 2015). Similarly, the integrin antagonist cilengitide did not improve the outcome in glioblastoma patients compared with radiotherapy, and the development of this drug was also discontinued (Stupp et al. 2014). Again the reason for these clinical failures remains unclear and are likely complex, including cross talk with diverse RTK signaling pathways.

An alternative to directly targeting cell surface receptors is identifying and targeting the signaling pathways that become activated downstream from cell surface receptors that mediate responses to multiple directional cues. Examples of such downstream targets toward which potent clinical inhibitors could be directed include FAK and Src kinase. Several clinical trials are ongoing that treat tumors by targeting FAK, although conclusive results are yet to be obtained. Interestingly, multiple preclinical studies have shown that inhibition of FAK may synergize well with therapies targeting other pathways or components of the microenvironment. Indeed, inhibition of FAK with VS-4718 delayed tumor progression in the p48-Cre;LSL-KrasG12D;Trp53flox/ KPC model for human pancreatic ductal adenocarcinoma (PDAC) and also rendered these tumors more sensitive to T-cell immunotherapy and PD-1 antagonists’ PDAC (Jiang et al. 2016). In metastatic melanoma, treatment with a FAK inhibitor prevented resistance to Braf inhibitors caused by fibroblast-mediated ECM deposition, which activated integrin signaling in tumor cells (Hirata et al. 2015).

Galvanotaxis, regulated mainly by VGSCs, can drive directional migration that contributes to metastasis (Frasier et al. 2005), suggesting that blocking VGSC activity can be a strategy to block metastasis. Indeed, inhibition of VGSCs by tetrodotoxin has been used in a prostate cancer in vivo model to reduce metastatic burden (Yildirim et al. 2012). Targeting pathways that allow cells to respond to EFs may be useful in targeting directional migration. Elucidating the mechanisms driving directional migration in response to multiple cues has increased our understanding of the pathways to target to inhibit metastasis. However, it is important to note how challenging it is to develop antimetastatic therapies from a clinical perspective (for a review, see Steeg 2016).

### Disrupting the Gradient

An alternate approach to target and prevent directional migration in tumors is by directly disrupting the endogenous gradients that drive tumor cell invasion (Fig. 5). Toward this objective, several stromal cells have been identified that can contribute to the generation of local growth factor or chemokine gradients that drive directional migration in tumors. The same stromal compart-

![Figure 5. Strategies to target directional migration.](image)
ment also plays an important role in the deposition of ECM, as well as its organization, and thereby regulates haptotaxis, alignotaxis, and durotaxis in tumors (Conklin and Keely 2012; Naba et al. 2012). Macrophages in particular, which secrete promigratory growth factors, were identified as viable therapeutic targets in cancer. However, despite these provocative findings, depletion of macrophages, through genetic or pharmacological perturbations, does reduce tumor cell motility and intravasation in breast cancer tumors (Wyckoff et al. 2004; Harney et al. 2015). Nevertheless, several pharmacological agents are currently in clinical trials that should block macrophage precursor recruitment, deplete tumor-associated macrophages, or reprogram their function that might prove more efficacious (Williams et al. 2016a). Inhibition of the CSF-1 receptor on macrophages in particular offers one promising strategy to reduce directed cell migration and tumor dissemination and is currently in clinical trials in advanced refractory breast or prostate cancer.

Oriented fibrillary collagen can promote directional migration in tumors through alignotaxis and durotaxis, suggesting strategies to prevent collagen alignment and stiffening, and may provide a viable therapeutic strategy to prevent tumor progression and metastasis. Consistent with this concept, several preclinical studies have attested to the utility of targeting collagen-processing enzymes to inhibit malignancy in vivo. For instance, inhibition of LOX through pharmacological inhibition or injection with lysyl oxidase (LOX)-neutralizing antibodies reduced tumor metastasis from solid tumors (Levental et al. 2009; Cox et al. 2013; Pickup et al. 2013). Similarly, studies using function-blocking antibodies to inhibit lysyl oxidase-like 2 (LOXL2) reduced collagen alignment and decreased tumor burden in a breast tumor xenograft (Grossman et al. 2016); these antibodies have been associated with invasiveness and poor outcome in breast cancer (Ahn et al. 2013). Higher LOXL2 levels are also associated with tumor invasion and poor outcome in pancreatic cancer patients and showed some efficacy in mouse models of PDAC (Park et al. 2016). However, when a LOXL2 inhibitor simtuzamab (Gilead) was tested in combination with gemcitabine in a cohort of previously untreated advanced pancreatic cancer patients, there was no observed significant clinical benefit over placebo. Alternately, FN is required for LOX-dependent collagen cross-linking, and treatment of mice with liver fibrosis with a peptide that blocks FN fibril formation (pUR4) significantly decreased their fibrosis, suggesting this type of treatment may be useful to reduce ECM accumulation in solid tumors (Altrock et al. 2015). Similarly, mice treated with the antifibrotic agent pirfenidone showed a significant reduction in lung metastasis (Takai et al. 2016).

Intriguingly, galvanotaxis has been extensively characterized in the context of wound healing (Zhao et al. 2006), and studies have shown that the movement of epithelial sheets can be easily and tightly controlled through manipulation of electric currents (Cohen et al. 2014). Such findings suggest that application of low-magnitude EFs could locally manipulate cell migration within tissues, particularly in the context of nonhealing and chronic wounds, and could be developed as a noninvasive approach to control the local invasive behavior of primary tumors (Wu and Lin 2014).

Taking Advantage of the Gradient

Chemotherapy remains the standard of care for most cancers despite its nonspecificity and high toxicity. One approach is to concentrate the chemical and target its delivery through microfabricated nanoparticles generated with diverse polymeric carriers that can be functionalized to specifically target the tumor itself by exploiting unique properties of the tumor microenvironment (Kwon et al. 2015). In this case, the inverted pH gradient present in a variety of solid tumors that acidifies the extracellular pH has been exploited by developing pH-sensitive carrier systems that permit optimal cargo release only in the presence of the low pH within the tumor (Tian and Bae 2012). For example, a layer-by-layer pH-responsive sheddable nanoparticle shell exists in which the neuronal layers that encapsulate the drug are only shed in response to the low pH in the tumor, exposing the nanoparticle surface loaded with a chemotherapeutic agent that can be taken up by the cells (Poon et al. 2011). Similarly, the hypoxic regions of the tumor have been leveraged to optimize therapeutic delivery to tumor regions using multiple mechanisms (Patel and Sant 2016). One such example is provided by a bioactive prodrug, such as the N-oxide drug tirapazamine (TPZ), which induces DNA damage, works synergistically with chemotherapy and radiation therapy, and is only activated under hypoxia (von Pawel et al. 2000). These data suggest that the exploitation of endogenous gradients (hypoxia, pH) within a tumor could be used to enhance drug-targeting strategies that would potentiate tumor treatment and reduce many undesirable side effects.

CONCLUSION AND OUTSTANDING QUESTIONS

How Do Different Gradients Affect Each Other?

Out of necessity, most cancer studies focus on the tumor cells responses to a single cue. Yet, in vivo, tumor cells simultaneously face a variety of endogenous gradients, many of which interact positively and negatively with each other (Fig. 6). This raises the important point that to understand tumor evolution it will be critical to design studies that can interrogate these complex physical and chemical interactions. For example, changes in ECM deposition and organization simultaneously promote haptotaxis, alignotaxis, and durotaxis. Furthermore, a recent study showed that GBM galvanotaxis proceeds toward the anode on a poly-L-ornithine/laminin 2D surface but switches to a cathode bias when the tumor cells are assayed with a 3D hyaluronic acid and collagen gel (Huang et al. 2016). This underscores the importance of using in vitro assays that can accurately model multiple aspects of the tumor microenvironment to gain a clear and accurate understanding of these complex cues.
understanding of the role of directional migration of tumors in vivo.

How Does One Cell Integrate Multiple Directional Cues?

The same cell surface receptors and signaling pathways have been shown to regulate directional responses to multiple cues, suggesting that there may be core signaling pathways that coordinate directional responses in several contexts. For example, the invasive isoform of the actin regulator MenaINV drives haptotaxis to high FN gradients (Oudin et al. 2016a) while also sensitizing cells to low EGF concentrations (Hughes et al. 2015). Interestingly, when breast tumor cells are incubated with both cues, expression of MenaINV renders them hyperinvasive, driving synergy between the signaling pathways downstream from both integrins and RTKs, leading to an increased migratory response that is stronger than each cue alone (Oudin et al. 2016b). Altogether, these studies highlight the importance of studying how different cues cooperate mechanistically to promote metastasis.

Attraction versus Repulsion?

In this review, we focused our discussion on how attractive cues promote local invasion. However, repulsive cues can also contribute to directional cell migration as has been consistently shown for neural guidance in the developing brain. For instance, the soluble ligand Slit and its transmembrane receptor Round-about (Robo) are well-established regulators of axon growth (Tessier-Lavigne and Goodman 1996); recent work suggests that this pathway may also play a role in cancer (Mehlen et al. 2011). This raises the intriguing possibility that repulsion may also play a role in modulating tumor cell guidance and metastasis.
How Do Current Therapies Affect Directional Migration?

Tumor cells that are particularly sensitive to physical and chemical directional cues are often the same cells that are intrinsically highly metastatic. This observation merits investigation because such heightened sensitivity could influence their treatment response and promote their dissemination. In this regard, Mena\(^{45,55}\), which drives chemotaxis and haptotaxis (Hughes et al. 2015; Oudin et al. 2016a), also promotes resistance to paclitaxel, a chemotherapeutic drug that is standard of care for the treatment of metastatic breast cancer (Oudin et al. 2017).

ACKNOWLEDGMENTS

This work was supported by K99-CA207866-01 to M.J.O. from the National Cancer Institute (NCI), the National Institutes of Health (NIH) NCI R01 grants CA138818-01A1, CA192914-01, CA174929-01, and CA085482 and 1U01CA202241-01 to V.M.W., and a Department of Defense (DOD) Breast Cancer Research Program (BCRP) grant BC122990 to V.M.W.

We apologize to the authors whose work we were unable to cite because of space limitations.

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