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## Hypoxia, inflammation, and the tumor microenvironment in metastatic disease

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### Abstract

Metastasis, the leading cause of cancer deaths, is an intricate process involving many important tumor and stromal proteins that have yet to be fully defined. This review discusses critical components necessary for the metastatic cascade, including hypoxia, inflammation, and the tumor microenvironment. More specifically, this review focuses on tumor cell and stroma interactions, which allow cell detachment from a primary tumor, intravasation to the blood stream, and extravasation at a distant site where cells can seed and tumor metastases can form. Central players involved in this process and discussed in this review include integrins, matrix metalloproteinases, and soluble growth factors/matrix proteins, including the connective tissue growth factor and lysyl oxidase.

### Keywords

Connective tissue growth factor; Lysyl oxidase; Metastasis; Hypoxia

## 1 Introduction

Cancer accounts for one in four deaths in the USA, corresponding to over 500,000 deaths in 2009. With more than 1,400,000 new cases diagnosed over the same time period in the USA alone, the gravity of the diseases we collectively call cancer is brought to light [1]. In order to better understand cancer and translate basic research into clinical treatments with improved patient outcomes, it becomes necessary to examine the component of cancer which causes the most deaths, metastatic spread of the disease, or metastasis. This review focuses on the metastatic process and the role of both tumor cell and extracellular matrix components that contribute to the progression of metastasis.

## 2 Cancer metastasis

The main cause of cancer death is metastasis of the primary tumor, which involves detachment of cells from the original tumor, invasion through the basement membrane, intravasation into the blood stream, and eventual extravasation from the blood stream at a

distant site where implantation and tumor cell proliferation give rise to cancer metastases. In order for this process to successfully transpire, there has to be remodeling of the extracellular matrix, changes in expression, and localization of key tumor-related and cytoskeletal proteins, proteases, and growth factors. Individual tumor cell and stromal components that are necessary for the metastatic process will be discussed within this section.

## 2.1 Epithelial to mesenchymal transition

Epithelial to mesenchymal transition, or EMT, was first coined by Krug et al. in 1987 but not characterized until 1995 [2, 3]. EMT is defined as the switch from non-motile, polarized epithelial cells to motile, non-polarized mesenchymal cells, with the potential to migrate from a primary tumor site to distant organs, at which they can seed and grow. While there is still debate as to whether EMT occurs in human patients, there is a breadth of literature describing this morphological alteration of cells *in vitro* and in mice *in vivo*, as well as changes in expression and localization of EMT markers, including E-cadherin, N-cadherin, Snail 1, Slug, and Twist [4–6]. A hallmark of EMT is a process termed the “cadherin switch,” defined by the loss of E-cadherin, the major component of adherens junctions, and a simultaneous gain of mesenchymal N-cadherin. This switch is characterized by dissolution of the adherens junctions, allowing cells to lose adhesive affinity for other epithelial cells and become more migratory and invasive [7, 8]. N-cadherin, which attaches to the cytoskeleton through interactions with both  $\alpha$ - and  $\beta$ -catenin, signals through the GTPases Rac1 and Cdc42, and interacts with the platelet-derived growth factor (PDGF) receptor, to initiate actin remodeling, thereby causing alterations in both cell adhesive properties and migratory status [9]. Partially responsible for this “cadherin switch” are transcriptional repressors of E-cadherin: Snail 1, Snail 2 (Slug), and Twist, which perform pro-invasive functions, such as inducing  $\alpha_v\beta_3$ -integrin expression, fibronectin, and matrix metalloproteinase-9 (MMP-9) [10]. In addition to transcriptional repressors, one of the most potent inducers of EMT is the transforming growth factor- $\beta$  (TGF- $\beta$ ), a known pluripotent growth factor able to induce EMT in mammary, lung, pancreatic, colon, and many other cell types [11–13]. While there are many studies examining individual components of EMT, the process as a whole still needs further defining both in an *in vitro* setting as well as in a clinical setting.

## 3 Tumor microenvironment

Under normal conditions, the cellular microenvironment is able to suppress malignant cell growth while tumor–stroma interactions modulate the microenvironment to be more permissive of malignant cell proliferation, motility, and adhesion. The tumor microenvironment is thought to be composed of endothelial cells, fibroblasts, perivascular cells, and inflammatory cells which regulate critical components of the tumorigenic process: angiogenesis, desmoplasia, lymphanogenesis, and inflammation, respectively. A specialized extracellular matrix, termed the basement membrane, separates epithelial and endothelial cells from stromal support components. It is composed of collagen IV, entactin, laminin, and heparin-sulfate proteoglycans, which confer tissue specificity, epithelial polarity, and functionality [14]. The basement membrane composition and structure is commonly altered

in cancer, along with changes in growth factor expression, recruitment of inflammatory cytokines, and increased fibroblast proliferation, all of which aid in the metastatic spread of tumor cells [15]. In addition to the tumor microenvironment being capable of influencing malignant cell growth by releasing ECM proteins, growth factors, and cytokines, tumors themselves secrete growth factors and proteases that are able to modify their local microenvironment making it more permissive for cell motility and adhesion.

### 3.1 Extracellular matrix

The extracellular matrix is a dynamic 3D structure composed of specialized proteins and proteoglycans whose interactions regulate many cellular processes, including cell survival, proliferation, differentiation, cell migration, and invasion. In order to better understand the extracellular matrix and its role in tumorigenesis, a few key extracellular matrix components, including integrins, matrix metalloproteinases, and soluble growth factors, such as lysyl oxidase (LOX) and the connective tissue growth factor (CTGF), will be discussed in the next few sections.

**3.1.1 Integrins**—Integrins are heterodimeric, type I transmembrane adhesion and signaling proteins, consisting of a combination of an  $\alpha$  and  $\beta$ -chain. There are 18 known  $\alpha$ -chains and 8  $\beta$ -chains, allowing for at least 24 unique heterodimers. When an integrin interacts with intracellular proteins, this causes a switch from an inactive, low affinity to active, high affinity state. Multiple heterodimer combinations allow for specific crosstalk with oncogenes and growth factor receptors on both tumor and tumor-associated cells, interactions with the extracellular matrix to provide traction necessary for cell motility and invasion, as well as assisting in matrix remodeling by directing localization of proteases [16]. Integrin expression varies between normal epithelial cells and tumor cells. Most notably,  $\alpha_v\beta_3$  expression is strongly correlated with tumor growth and metastasis of breast, prostate, pancreatic, glioblastoma, cervical, and ovarian cancers [17–23]. Integrins are effectors of signaling cascades including MAP kinase, Jun, NF $\kappa$ B, and  $\beta$ -catenin as well as direct downstream targets of Src-family kinases, focal adhesion kinase (FAK) and protein kinase B [9]. Interactions with these signaling cascades allow integrins to modulate cell survival, proliferation, cell migration, and invasion. Since certain integrins, including  $\alpha_v\beta_3$ , are expressed on both tumor and angiogenic endothelial cells, they would make good therapeutic targets. Indeed, many clinical studies are underway, including one with etaracizumab, an early clinical integrin agonist. Additionally, an integrin-specific monoclonal antibody, CNTO 95, that targets both  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins, has demonstrated anti-tumor activity in initial phase I trials [24]. Another set of therapies specifically target  $\beta_1$  integrins, an example of this is volociximab, a function-blocking antibody against  $\alpha_v\beta_1$ . In phase I trials, it was well tolerated and demonstrated possible clinical efficacy in patients with solid malignancies [16, 25]. Additional integrin agonists are currently in phase II and phase III clinical trials [16].

**3.1.2 Matrix metalloproteinases**—Matrix metalloproteinases are members of a family of endopeptidases critical to the tumor microenvironment and process of EMT. The first member of this family was discovered in 1962 through its ability to degrade collagen during tadpole tail metamorphogenesis [26]. Since then, 24 human family members have been

characterized. They comprise a collection of zinc-dependent endopeptidases that function at physiological pH and, at low levels, regulate normal remodeling of human connective tissue as well as being involved in ovulation. These metalloproteinases are secreted as inactive proteins, through an association between a conserved cysteine within the pro-domain, “Pro-Arg-Cys-Gly-X-Pro-Asp,” and a zinc within the catalytic site [27]. Proteolytic cleavage is necessary for full enzymatic activity. The family is divided into subclasses based on their substrates: collagenases, stromelysins, matrilysins, and gelatinases [28]. Once activated, the majority of MMPs function to degrade collagen (types I, II, and III), the most abundant protein in the human body accounting for nearly 30% of the total, as well as liberating growth factors and peptides anchored in the extracellular matrix. More specifically, MMP-13 is unique to collagen type II degradation in cartilage, while MMP-2 and MMP-9 degrade type IV collagen, and MMP-1 is robustly expressed and degrades all three types of collagen [29]. Under normal disease-free conditions, MMPs tend to be expressed at low levels and are kept in check by tissue inhibitors of metalloproteinases (TIMPs). This family consists of four protease inhibitors, which function to block MMP activity at a 1:1 molar ratio by forming a complex with the activated catalytic zinc in the MMPs [27]. During disease pathogenesis, MMP levels often rise more quickly than TIMPs, causing MMP activation and remodeling of the tumor microenvironment as well as activation of cell surface receptors and their downstream signaling cascades. MMP-2 and MMP-9 are commonly linked to the process of EMT because they are known to degrade type IV collagen, the main component of the extracellular matrix on which the epithelium resides. Numerous studies have examined the expression of MMPs in disease progression, and consistently, MMP-2 and MMP-9, as well as other family members, have been shown to be increased in patients with advanced stage breast, lung, pancreatic, prostate, ovarian, and colorectal cancers and are often correlated with decreased survival [28, 30–32]. In addition to being valuable biomarkers, MMP inhibitors (MMPIs) are being examined as potential tools to be utilized in a clinical setting. While initial studies utilized broad spectrum inhibitors causing off-target effects, more recent studies have been taking advantage of structural knowledge to produce targeted MMP inhibitors [33]. For example, one study took this target-based approach and discovered a series of non-zinc chelating compounds with potent MMP-12 affinity in the nanomolar range [34]. While there is a lot of convincing literature supporting the role of MMPs in metastatic progression, more work needs to be done to examine whether MMPIs will be clinically effective.

### 3.2 Hypoxia

A key environmental stressor associated with tumor progression and poor clinical prognosis is tumor cell oxygen deficiency, termed hypoxia [35]. This condition is known to induce genes involved in the regulation of cell proliferation, extracellular matrix production, cell adhesion, and other hallmarks of tumorigenesis. The mechanism behind these effects is frequently accomplished through induction of the hypoxia-inducible factor (HIF) family of transcription factors. This family consists of three members, HIF-1, -2, and -3, which act to regulate cellular processes including glucose metabolism, angiogenesis, cell proliferation, and tissue remodeling in response to low oxygen levels. Under normal oxygen conditions, a group of prolyl-4-hydroxylases (PHDs) hydroxylate HIF-1 $\alpha$  on two conserved residues, proline 402 and proline 564 [36–38]. This allows recognition HIF-1 $\alpha$  by the von Hippel-

Lindau (VHL) tumor suppressor. VHL functions as an E3 ubiquitin ligase and modifies HIF-1 $\alpha$  by adding ubiquitin ladders, targeting it to the proteasome for degradation. Under conditions of acute or chronic hypoxia, HIF-1 $\alpha$  is stabilized in an oxygen-dependent manner because the hydroxylation and subsequent degradation of HIF-1 $\alpha$  by PHDs requires oxygen and 2-oxyglutarate as substrates, as well as iron and ascorbate as cofactors [39, 40]. Once stabilized, HIF-1 $\alpha$  can form a heterodimer with HIF-1 $\beta$ , allowing this transcription factor to bind a core sequence of 5'-RCGTG-3' and increase transcription of target genes [41].

While hypoxia has been strongly linked to tumor metastasis and poor clinical outcome for patients, it seems to actually have a dual role: insufficient oxygen limits tumor cell division while at the same time selecting for more malignant cells and inducing cell adaptations allowing for more invasive behavior. Regarding tumor growth, cancer cells, similar to normal cells, need oxygen to generate energy as well as acting as a substrate for many fundamental cellular processes, including generating macromolecules. Yet, hypoxia is also strongly associated with tumor progression and metastatic disease. This is likely because low oxygen tension is able to increase cell invasiveness, cause cells to switch to anaerobic metabolism, increase genetic instability, and promote angiogenesis [42–47].

The ability of tumor cells to invade is a critical step in metastasis. There are several methods through which hypoxia can cause an increase in metastatic potential of tumor cells. One way is through HIF-1 $\alpha$  binding to hypoxia-response elements within the c-met promoter activating transcription of this gene. Overexpression of the Met protein on the cell surface of tumor cells leads them to be more susceptible to hepatocyte growth factor stimulation. This causes extracellular matrix degradation, cell dissociation, and escape from hypoxic areas to more oxygen-rich environments at a secondary site [46]. Additionally, a study in renal cell carcinomas demonstrated that hypoxia-induced HIF expression is necessary and sufficient to cause E-cadherin loss, a critical step in EMT [48, 49], while yet another study showed that activation of the Wnt/beta-catenin signaling pathway, through HIF-1 $\alpha$ , can induce prostate cancer cells to be more motile and invasive [50]. Moreover, expression of hypoxia-induced factors, such as lysyl oxidase (discussed in further detail below), can cause cancer cells to favor metastatic spread as demonstrated in both *in vitro* and *in vivo* experiments (Fig. 1) [51].

Angiogenesis, another critical step in tumorigenesis, is defined by the formation of new blood vessels which provide nutrients to the growing tumor as well as an escape route for cancer cells to travel to distant sites in the body. Due to the growing demands of solid tumors, invariably their vasculature is impaired with a lack of strong interconnections and pericytes, leakiness, and transient changes in oxygenation [52, 53]. Hypoxia, through HIF-1 $\alpha$ , induces the secretion of pro-angiogenic factors including vascular endothelial growth factor (VEGF), angiopoietin 2 (Ang-2), PDGF, and fibroblast growth factor [54–57]. In addition to these factors, HIF-1 works to decrease the activity of angiogenic inhibitors, such as thrombospondin, thereby creating a pro-angiogenic environment [58].

Clinically, both HIF-1 $\alpha$  and HIF-2 $\alpha$  are increased in tumors, including renal, breast, colon, and pancreatic cancers [59]. With oxygen deprivation having such a large effect on many steps of the metastatic cascade, targeting hypoxia for human therapies makes sense. But

hypoxia poses a variety of problems in the treatment of cancers by decreasing the effectiveness of chemotherapy and radiotherapy [35, 60]. The majority of cytotoxic chemotherapies require proliferating cells to induce cytotoxicity. However, tumor cells in regions of hypoxia divide slower, and therefore, chemotherapies are less effective in the hypoxic regions of solid tumors. Initial treatments to target hypoxia include hyperbaric oxygen (intermittent administration of 100% oxygen at high pressure), as well as systemic erythropoietin treatment [61, 62]. A recent study which utilized genetic manipulation of the hypoxic tumor environment by ectopically expressing myoglobin (Mb) in human A549 lung cancer cell xenografts proved more successful. Myoglobin is a cytoplasmic protein involved in oxygen transport and free radical scavenging. It is thought that Mb can decrease HIF-1 $\alpha$  stabilization while allowing cancer cells to utilize oxygen more effectively in a hypoxic environment resulting in delayed engraftment, tumor growth, and microvascular density *in vivo* [63].

**3.2.1 Lysyl oxidase**—Lysyl oxidase, or LOX, is a copper-dependent amine oxidase that cross-links collagen and elastin molecules in the extracellular matrix [64]. This highly conserved protein was discovered in 1968 and is part of a larger family consisting of five members: LOXL, LOXL2, LOXL3, LOXL4, and LOX [65]. It is translated as a 48-kDa preproprotein which is subject to cleavage of its N-terminal signal peptide and is glycosylated prior to secretion. Once secreted, the catalytically inactive pro-enzyme protein is further cleaved by extracellular proteases releasing the 32-kDa mature and catalytically active LOX enzyme [66–68]. In addition to being found extracellularly, mature LOX has also been found to be localized to the nucleus in a NIH3T3 cell culture system, suggesting nuclear activity [69, 70]. Indeed, intranuclear LOX can interact with histone H1 and is therefore implicated in modifying gene expression [71, 72]. The collagen and elastin cross-links formed by this enzyme lead to increases in tensile strength and structural integrity necessary for normal connective tissue function, embryonic development, tissue remodeling, and repair of diseased and aging connective tissues [73, 74]. LOX<sup>-/-</sup> mice die shortly after birth due to structural and functional defects in the cardiovascular system, lungs, and skin [75, 76]. Localization of LOX occurs in extracellular compartments within many regions of the body, including skin, liver, heart, lungs, skeletal muscle, cartilage, and kidney and pancreas in mammals [70, 77, 78].

In human disease, there have been numerous studies linking LOX to tumorigenesis. Currently, LOX is the only tumor-secreted factor shown to be directly involved in pre-metastatic niche formation. Increased LOX expression correlating to disease progression, metastasis, and poor overall survival has been shown in breast cancer, head and neck cancers, as well as oral cancers [51, 79–81]. LOX expression is associated with hypoxia and is increased in hypoxic tumors (as determined by CAIX expression) in breast and head and neck cancers [82]. Additionally, LOX expression coincides with hypoxia and correlates to decreased metastasis-free and overall survival in patients. *In vitro* studies have demonstrated a role for LOX in invasion of human cutaneous melanoma and breast cancer cell lines as well as cell migration through its regulation of FAK activity [51, 83]. LOX can also regulate cell adhesion through increased cyclin D1 and  $\beta$ -catenin expression [84]. Furthermore, the

catalytic domain of LOX and LOX family members has been shown to bind Snail, suggesting that these proteins play a role in EMT [85].

Since LOX expression and activity regulate a number of important steps in the metastatic process, there are studies examining its potential as a therapeutic target. Utilizing a specific antibody to LOX in an *in vivo* orthotopic breast cancer model, lung and liver metastases were dramatically decreased while primary tumor growth was unaffected [51]. This demonstrates the importance of LOX in late-stage metastatic disease. Further studies examining the mechanisms behind LOX's contribution to the metastatic process and its potential as a therapeutic target still need to be investigated.

**3.2.2 Connective tissue growth factor**—Another hypoxia-regulated extracellular matrix protein is CTGF, or connective tissue growth factor. CTGF is a transcriptional target of HIF-1, which directly interacts with HREs located in the 5' region of the protein [86]. It is a member of the CCN family of secreted proteins, which are cysteine-rich matricellular proteins composed of four modular domains with homology to insulin-like growth factor-binding proteins (domain 1), a von Wille-brand factor type C repeat (domain 2), a thrombospondin type 1 repeat (domain 3), and a cysteine-knot domain (domain 4) [87].

While CTGF's function in normal tissue has been well studied, the knowledge of its role in tumor biology is still being discovered. It is known that CTGF exerts a dual role as both a tumor suppressor and tumor promoter, depending on the cancer context. Specifically, the expression of CTGF has been demonstrated to be increased during tumor growth in many cancers including pancreatic [88], glioblastoma [89], and melanoma [90]. Both CTGF messenger RNA (mRNA) and protein have been shown to be induced in response to hypoxia in a human breast cancer model *in vitro* [91]. Studies examining human pancreatic specimens also displayed a 40- to 50-fold increase in CTGF mRNA expression in tumor compared to normal samples [88, 92]. Additionally, CTGF co-localized with regions of hypoxia in human tumor xenografts as well as clinical pancreatic adenocarcinoma specimens [93]. CTGF secretion was also increased in hypoxic tumor cells *in vitro* [93]. In an *in vivo* Panc 1 model of pancreatic cancer, subcutaneous and orthotopic tumor growth was found to be critically dependent on CTGF being expressed by the tumor cells. In addition, CTGF expression increased anchorage-independent growth of pancreatic tumor cells in soft agar while decreasing apoptosis in response to hypoxic stress [93]. Similar results were found in another model of pancreatic cancer utilizing MiaPaca2 cells, where CTGF was able to promote growth in soft agar as well as *in vivo* primary tumor growth. A neutralizing CTGF-specific monoclonal antibody, FG-3019, successfully inhibited both anchorage-independent growth *in vitro* as well as primary and lymph node metastasis *in vivo* [94]. These studies demonstrate the importance of CTGF as a potential therapeutic target, especially in pancreatic cancer. Additional studies have shown CTGF to be upregulated during inflammation, fibrotic disorders, and angiogenesis, all critical factors for primary tumor growth and metastasis [95–98]. It is important to note that while CTGF expression generally correlates with decreased survival, exceptions have been found in esophageal squamous cell carcinoma and chondrosarcoma [99, 100].

### 3.3 Inflammation

Another aspect of the tumor microenvironment that has a significant role in neoplastic progression is inflammation. Under normal conditions, such as wound healing, an influx of cytokines and chemokines work in a self-limiting approach to heal the wound. But dysregulation of this process can lead to abnormalities in the inflammatory response and ultimately tumorigenesis. Worldwide, approximately 1.2 million cancer cases are thought to arise from chronic inflammation and infections [101, 102]. One of the best examples of this is colon cancers which often develop following inflammatory bowel disease. Inflammation in carcinomas involves different factors than what is seen with a typical inflammatory response. For example, an influx of pro-inflammatory cytokines, including tumor necrosis factor- $\alpha$  and TGF- $\beta$ , as well as cytotoxic mediators, proteases, MMPs, interleukins, and interferons, produce potent lymphangiogenic and angiogenic growth factors allowing tumor growth and metastatic spread to the lymph nodes [103, 104]. More specifically, tumor-associated macrophages produce pro-angiogenic growth factors, such as vascular endothelial growth factors (VEGF-C, -D) and VEGF receptor 3. Tumor cells themselves produce cytokines which attract neutrophils, macrophages, lymphocytes, and dendritic cells all attributing to tumorigenic growth and metastatic potential [105].

## 4 Conclusions

Since metastasis is the main cause of death from cancer, exploring the sources of metastatic disease is critical for the development of therapies to be used clinically. A great deal of research has focused on the tumor microenvironment and the role of extracellular matrix proteins in disease progression. This review highlighted the importance of integrins, matrix metalloproteinases, and extracellular matrix proteins, such as LOX and CTGF, and their roles in epithelial to mesenchymal transition. Additionally, this review also covered the importance of oxygen deprivation and inflammation on tumorigenesis. As more components of the metastatic cascade are discovered and interactions between known extracellular matrix proteins and tumor-expressed proteins are brought to light, new classes of therapeutic targets will emerge.

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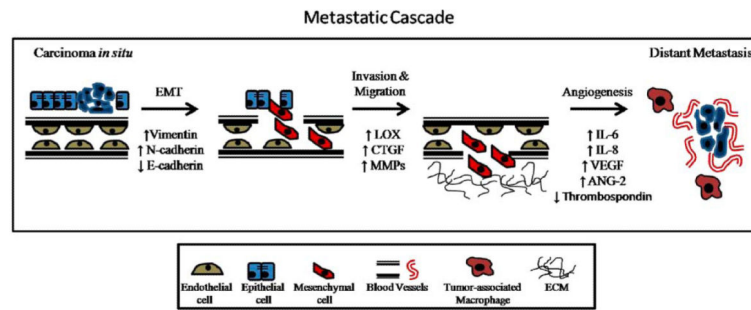
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**Fig. 1.**

Role of hypoxia and inflammatory-regulated genes on metastasis. The process of metastasis often occurs in regions of oxygen deprivation, termed hypoxia, in which HIF can induce expression of pro-invasive and pro-angiogenic factors, such as LOX, CTGF, VEGF, Ang-2 and decreases in E-cadherin and thrombospondin. Additionally, the inflammatory response induces tumor-associated macrophages which stimulate pro-angiogenic factors, including IL-6, IL-8, and VEGF. Together, hypoxia and inflammation promote an environment permissive of metastatic disease