Chapter 3
Hypoxia-Mediated Metastasis

Joan Chang and Janine Erler

Abstract Metastasis is responsible for more than 90 % of deaths among cancer patient. It is a highly complex process that involves the interplay between cancer cells, the tumor microenvironment, and even noncancerous host cells. Metastasis can be seen as a step-wise process: acquisition of malignant phenotype, invasion into surrounding tissue, intravasation into blood vessels, survival in circulation, extravasation to distant sites, and colonization of new organs. Before the actual metastatic process, the secondary site is also prepared for the arrival of the cancer cells through formation of "premetastatic niches." Hypoxia (low oxygen tension) is commonly found in solid tumors more than a few millimeters cubed and often is associated with a poor prognosis. Hypoxia increases angiogenesis, cancer cell survival, and metastasis. This chapter described how hypoxia regulates each step of the metastatic process and how blocking hypoxia-driven metastasis through targeting hypoxia-inducible factor 1, or downstream effector molecules such as the lysyl oxidase family may represent highly effective preventive strategies against metastasis in cancer patients.

Keywords Hypoxia • Metastasis • Extracellular matrix (ECM) • Epithelial-mesenchymal transition (EMT) • Microenvironment • Angiogenesis

3.1 Metastasis

Solid tumors, regardless of organ type and cell of origin, can be described as either benign or malignant. Benign tumors remain localized and lack the ability to escape the primary tumor site, whereas malignant tumors can spread through invasion and

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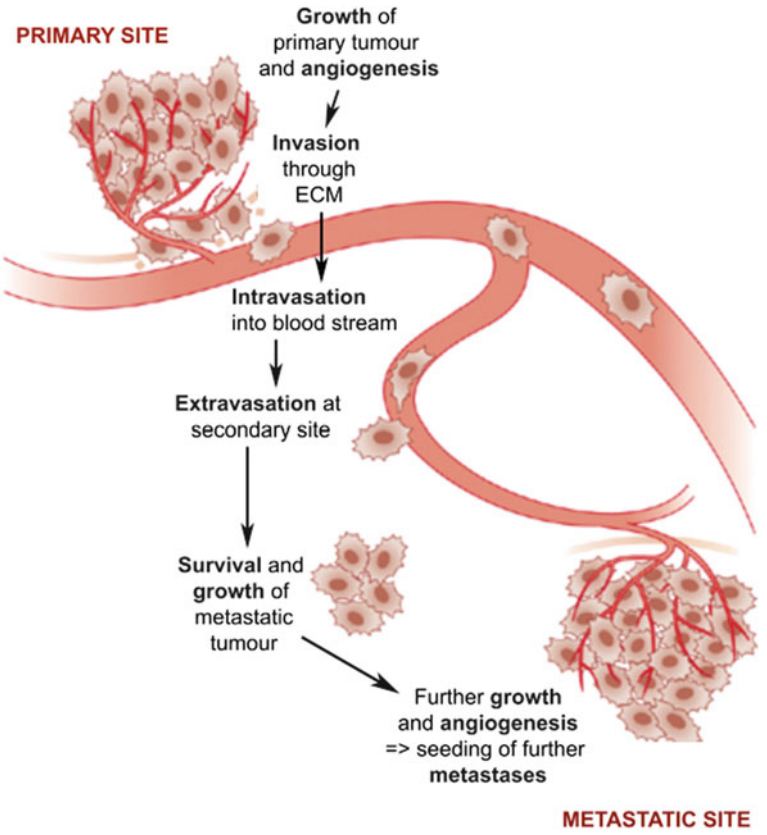


Fig. 3.1 The multistep process of metastasis. The metastatic process consists of a series of distinct, sequential steps, each of which must be achieved for successful metastasis. Adapted from Oncology News Volume 6 Issue 4, 2011. http://www.oncologynews.biz/pdf/sep_oct_11/128-131_ONSO11_feature%20art.pdf

metastasis of the cancer cells. Initial cancer research mostly focused on investigating the molecular basis of oncogenic transformation, which gives rise to (primary) tumors; relatively less is known about the process by which tumor cells become metastatic and colonize distant organs. However, focus has recently shifted to understanding the metastatic process because metastasis remains the cause of more than 90 % of deaths among cancer patients with solid tumors (Gupta and Massague 2006).

Metastasis is considered one of the original six acquired hallmarks of cancer (Hanahan and Weinberg 2000, 2011). It is generally believed to be a multistep process (Fig. 3.1) consisting of discrete biological processes. To escape from the primary tumor, cancer cells first disrupt the integrity of the basement membrane (BM), then invade the surrounding interstitial extracellular matrix (ECM), and intravasate into the circulatory system. The cancer cells then must survive the fluctuating environment in transit as circulating tumor cells (CTCs), and extravasate to distant sites as disseminated tumor cells (DTCs) that invade into and colonize new organs within

the body. The final step of metastasis is acquiring vasculature to support the growth of the metastases; in some cases, these metastases can repeat the whole process to give rise to new metastases.

3.2 Hypoxia and Metastasis

Hypoxia has been shown to decrease the efficacy of radiation therapy (Overgaard and Horsman 1996), and because of the poor vasculature support, the efficiency of drug delivery to hypoxic tumor cells is greatly reduced (Chaudary and Hill 2007). In addition, tumor cells in regions of hypoxia undergo slower cell division and have decreased apoptotic potential – thus chemotherapies are less effective (Erler et al. 2004; Finger and Giaccia 2010). However, the clinical effect of hypoxia on cancer biology extends beyond its effects on therapeutic efficacy.

The presence of tumor hypoxia is associated with poor survival and increased metastatic incidence and burden in patients with various cancer types, including head and neck, cervical, and breast (Hockel and Vaupel 2001; Harris 2002; Cairns et al. 2003; Pouyssegur et al. 2006). In one of the earliest clinical studies, a computerized polarographic electrode system was used to investigate the tumor oxygenation in locally advanced cancer of the uterine cervix over a period of 8 years, where tissue pO_2 partial pressure of oxygen in the blood was measured in patients with cervical tumors ≥3 cm in diameter (Hockel et al. 1996). The study showed that 52 patients with hypoxic tumors (median pO_2<10 mmHg) had worse disease-free and overall survival, and in patients in whom the primary tumor was surgically removed there was greater incidence of distant metastases if the primary tumor was hypoxic (Hockel et al. 1996). A more recent study showed hypoxia to be a prognostic marker of distant disease recurrence in 106 node-negative patients with cervical cancer and that tumor hypoxia (pO_2<5 mmHg in this case) can be used to predict progression-free survival of these patients (Fyles et al. 2002). Moreover, patients with hypoxic tumors also had a significant increase in the incidence of distant metastases when compared to patients with more oxygenated tumors (Fyles et al. 2002).

Hypoxia plays a dual role in cancer progression: on the one hand it limits the primary tumor growth because cancer cells require oxygen for fundamental cellular processes; on the other hand hypoxia selects for more invasive cells and thus promotes malignant progression – as one can imagine the tumor cells would want to physically move toward an oxygen-rich environment. It is interesting to note that hypoxic tumor cells may be found not only toward the center of a primary tumor mass but also at the invasive front, highlighting the dynamic nature of tumor hypoxia (Buchler et al. 2004).

The cellular response to hypoxia is predominantly mediated by the helix-loop-helix transcription factor hypoxia-inducible factor (HIF)-1. HIF-1, a heterodimer composed of one of three alpha subunits (HIF-1α, HIF-2α, HIF-3α) and one beta subunit (HIF-1β), is active under hypoxia by the stabilized expression of the alpha subunit. The HIF-1α and HIF-2α subunits in particular are overexpressed and

associated with poor prognosis in many cancer types (Semenza 2003; Qing and Simon 2009). Moreover, HIF-1α is expressed at a higher fraction (69 %) in metastases compared with primary tumors (29 %) (Zhong et al. 1999), and patients with higher proportions of hypoxic cells have decreased disease-free and overall survival rates after surgical resection of the primary tumor due to the recurrence of metastatic disease (Hockel et al. 1996; Vergis et al. 2008). These two observations strongly link hypoxia with the metastatic progression of cancer. Studies of hypoxia-regulated genes have revealed upregulation of genes involved in multiple biological functions that strongly influence metastatic progression, such cell proliferation, angiogenesis, and ECM remodeling (Table 3.1) (Le et al. 2004; Rankin and Giaccia 2008).

3.3 Rise of the Metastatic Population

Primary tumor cells may become metastatic in various ways; however, we focus on three main possibilities. The first possibility suggests that Darwinian evolution selection pressures may have been present in the primary tumor environment, selecting for cancer cells that have acquired aggressive phenotypic traits through genetic or epigenetic changes, thus allowing for clonal expansion of this "fitter" cell type. The fitter populations are eventually (as well as inevitably) able to disseminate to secondary sites. Metastatic events in this case rely on the "nature" of the primary tumor cells. Hypoxia is known to select for this type of fitter population. Paradoxically, while hypoxia is usually lethal for most normal cell types, hypoxia selects for cancer cells with low apoptotic potential (Graeber et al. 1996; Erler et al. 2004) and increases genomic instability, which in turn allows cancer cells to rapidly mutate and adapt to the microenvironment and more quickly acquire aggressive traits (Young et al. 1988; Reynolds et al. 1996). In addition, hypoxia can also induce cancer cells to secrete various growth factors and proteases to alter their immediate microenvironment, thereby permitting invasion and promoting angiogenesis.

The second possibility suggests that the primary tumor cells have enhanced survival and proliferative abilities but have not yet acquired the aggressive traits that allow for invasion and metastasis. In this case, the metastatic events occur because of the tumor cells responding to contextual signals provided by the tumor microenvironment. In normal cellular microenvironments, malignant cell growth is suppressed; the tumor microenvironment (including hypoxia), however, promotes invasion and metastasis of the cancer cells.

The third possibility in a way unites the first two possibilities, suggesting the existence of metastatic cancer stem cells that are the fitter population, as described in the first possibility. These are thought either to be present right from the beginning or are cancer stem cells (CSCs) modulated by the tumor microenvironment in such a way that makes them metastatic, as described in the second possibility. Recent studies revealed that within a tumor, populations of cells are organized in a hierarchy, recapitulating the scheme of self-renewing stem cells, progenitor cells, and fully differential cells found in normal tissues (Bonnet and Dick 1997; Al-Hajj et al. 2003; Ailles and Weissman 2007) and suggesting the presence of CSCs that

Table 3.1 Overview of reported hypoxia-regulated genes and their respective roles in cancer progression

GLUCOSE TRANSPORTATION/ METABOLISM
Acetoacetyl CoA thiolase
Adenylate kinase-3
Aldolase A,C
Aminopeptidase A
Cabonic anhydrase-IX, -XII
Ceruloplasmin
Enolase-1
Erythropoietin
Ferritin light chain
Fructose-2,6-bisphosphatase-3
GLUT-1, -3
Glyceraldehyde-3-phosphate dehydrogenase
Glycogen-branching enzyme
Heme oxygenase
Hexokinase-1, -2
Lactate dehydrogenase A, B
Max interactor-1
Phosphofructokinase L
Phosphoglycerate kinase-1
Phosphoribosyl pyrophosphate synthetase
Pyruvate dehydrogenase kinase-1
Pyruvate kinase-M
Solute carrier family
Spermidine N1-acetyltransferase
Transferrin and receptor
Transglutaminase-2
Triose phosphate isomerase
Tyrosine dehydroxylase
6-Phosphofructo-2 kinase

PROLIFERATION/ DIFFERENTIATION
Adipophilin
B-cell translocation gene-1
Cyclin-dependent kinase inhibitor-1B (p27, kip1)
Cyclin D1, G2
Cyclin-dependent kinase-1
Cyclin-dependent kinase inhibitor-1 (p21)
Deleted in esophageal cancer-1 (DEC1)
Erythropoietin
Inhibitor of DNA binding-2
IGF-2
IGF-binding protein-2
Mitogen-inducible gene-6
N-myc downstream regulated gene-1 (Cap43)
Stimulated by retinoic acid-13 (stra13)
TGF-α

ANGIOGENESIS
Adrenomedullin
Angiopoietin-1, -2
Angiopoietin-1 receptor (TIE-2)
Cyclooxygenase-1, -2
Endothelin-1, -2
Ephrin A1
Fibroblast growth factor-3
Hepatocyte growth factor
Matrix metalloproteinase-2, -9
Nitric oxide synthase
Placental growth factor
Plasminogen activator inhibitor-1
PDGF-B
Thrombospondin-1, -2
TGF-α, -β1, -β3
VEGF-A, -B, -C, -D
VEGF receptor 1 (FLT-1)
VEGF receptor 2 (FLK-1)

GROWTH FACTORS/ CYTOKINES
IGF-2
Interleukin-6, -8
Intestinal trefoil factor
Macrophage migration inhibitory factor
PDGF-B
Stanniocalcin-2
TGF-α

APOPTOSIS
BCL-w like
BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3)
BNIP3-like
Hepatic fibrinogen/angiopoietin-related protein
IGF-binding protein-1, -3, -5
Proto-oncogene serine/threonine-protein kinase (PIM)-1, -2
RTP801 (REDD1)

STRESS-RESPONSE
Growth arrest and DNA damage inducible gene (GADD)-153
Heat shock factors
Heat shock proteins
Huntington-associated protein-1
Hypoxia upregulated protein 1 (ORP150)
Thioredoxin

TISSUE REMODELING
c-MET
CD99
CXCR type-4
Collagen-5a
Galectin-1
Integrin-5a
Ku70
Low-density lipoprotein receptor-related protein
LOX
LOX-like 2
Lysyl hydroxylase-2 (PLOD2)
MMP-7, -13
Mucin 1
Osteopontin
Plasminogen activator inhibitor-1
Prolyl-4-hydroxylase
Tissue factor
UPAR
Vimentin

GENE EXPRESSION
Early growth response 1
P35srj
ETS-1
Mxi-1
Annexin V
BCL-interacting killer
FOS
Jun
Lipocortin
Nuclear factor κB
NIX
NR3C1 glucocorticoid receptor-α
Nuclear factor IL-3

INVASION/METASTASIS
Connective tissue growth factor
CXCR type-4
E-cadherin
LOX
LOXL2
PAI-1
Stromal-derived factor-1
UPAR

CoA coenzyme A; *CXCR* C-X-C chemokine receptor; *GLUT* glucose transporter; *IGF* insulin-like growth factor; *LOX* lysyl oxidase; *TGF* transforming growth factor; *PDGF* platelet-derived growth factor; *UPAR* urokinase plasminogen activator receptor; *VEGF* vascular endothelial growth factor

have self-renewal properties and enhanced tumor-initiating potential. The definition of a CSC in research is that the cancer cell has the capacity to self-propagate to form new tumors when experimentally implanted into animal hosts. This is, in theory, similar to tumor initiation at secondary sites by DTCs; both scenarios require the "seeder" to have the ability to self-renew and initiate colonization by producing progenies. As such, each metastatic cell of origin is thought to be from a DTC with CSC properties. Thus, CSC phenotype–enhancing mechanisms should increase the efficiency of metastasis. Hypoxic regulation of stem cells is covered in Chap. 2.

Regardless of how the metastatic population arises, it is known that hypoxia greatly increases the invasive and metastatic potential of cancer cells. Hypoxia is thus considered a potent driving force in the prometastatic microenvironment, and it influences each stage of the metastatic process, as detailed in the following sections.

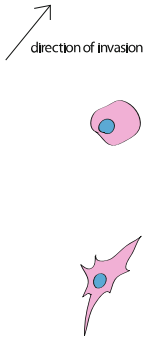
3.4 Cancer Cell Invasion

Invasion is the first step of metastasis in which the cells invade various biological barriers to get into blood vessels for dissemination. In addition to breaking down physical barriers, the invasive cells can also acquire changes in their cell-cell and cell-matrix adhesion interactions.

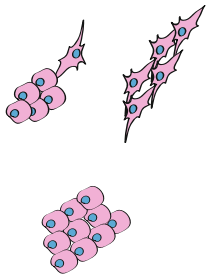
3.4.1 Hypoxia and the Epithelial-Mesenchymal Transition

Investigations into cancer cell movement (migration) have revealed that tumor cells can migrate individually or collectively as a group (Table 3.2). Single-cell migration takes the form of either amoeboid, leukocyte-like or mesenchymal migration (Friedl and Gilmour 2009; Madsen and Sahai 2010). Amoeboid-like migration allows the cancer cells to migrate through the stroma without the need to proteolytically remodel the matrix around them, often "hitching a ride" along collagen fibers in the ECM (Condeelis and Segall 2003). Mesenchymal migration, on the other hand, is thought to require cancer cells to undergo epithelial-mesenchymal transition (EMT). Cancer cells undergoing EMT lose their cell-cell adherent properties and polarity, acquire an invasive mesenchymal phenotype, and become resistant to apoptosis and senescence (Thiery et al. 2009; Yilmaz and Christofori 2010). EMT cancer cells can migrate either individually or collectively with cells that have undergone EMT at the front, clearing out a track through which follower cells can move (Erler and Giaccia 2006; Friedl and Wolf 2008). This requires proteolytic degradation of the various components of the biological barriers, which involves various protein families such as the matrix metalloproteinase (MMP) family, the adamalysin-related membrane proteinases, and tissue serine proteinases (Andreasen et al. 2000; Sternlicht and Werb 2001). Interestingly, collectively migrating cancer cells may still retain epithelial characteristics by either hitching a ride with the invasive EMT cells or following migrating host stromal fibroblasts (cancer-associated fibroblasts, CAFs)/tumor-associated

Table 3.2 Mechanisms of cancer cell migration



INDIVIDUAL	COLLECTIVE
Ameboid (e.g. lymphoma, small cell lung cancer, leukemia)	**Clusters** (e.g. epithelial cancers, melanoma)
Mesenchymal (e.g. fibrosarcoma, glioblastoma, anaplastic tumours)	**Sheets** (e.g. epithelial cancers, vascular tumours)



macrophages (TAMs) (Condeelis and Pollard 2006; Gaggioli et al. 2007; Joyce and Pollard 2009), which are described in more detail in the next section.

Nonetheless, the clinical relevance of EMT is unknown and it is still unclear how much tumors depend on EMT for metastatic progression. However, experimental studies clearly show the benefit of EMT in cancer progression (Iwatsuki et al. 2010; Tsai et al. 2012). EMT is typically represented by a loss of the epithelial cell marker E-cadherin, which facilitates cell-cell adhesion, and induction of the mesenchymal cell marker N-cadherin, which facilitates cell-matrix adhesion (Lee et al. 2006). It is well established that hypoxia can directly induce EMT through HIF-1, upregulating the expression of various EMT-activating transcription factors: Twist-related protein 1 (Twist 1, also known as Twist), zinc finger protein Snai1 (SNAI1), zinc finger E-box-binding homeobox 1/2, and transcription factor 3 (Imai et al. 2003; Krishnamachary et al. 2006; Yang et al. 2008).

It is interesting to note that another hypoxia-induced ECM protein family – the lysyl oxidase (LOX) family (Fig. 3.2) – has been shown to play a key role in the EMT process, in particular the members LOX and LOX-like 2 (LOXL2). The *LOX* and *LOXL2* genes are targets of HIF-1 and may play a role in EMT through both their reported intracellular roles and extracellular roles (Schietke et al. 2010). The enzymatic function of extracellular LOX has been shown to stimulate Twist transcription, thereby mediating the EMT of cancer cells (El-Haibi et al. 2012). LOXL2, on the other hand, is involved in the regulation of epithelial cell motility through HIF1 and hypoxia (Higgins et al. 2007), and intracellular LOXL2 has been shown to interact with and stabilize SNAI1, thus inducing EMT (Peinado et al. 2005). However, it also has been shown that while LOXL2 influences SNAI1-dependent invasive properties in cancer cells, LOXL2 also has specific SNAI1-independent functions in cancer progression (Peinado et al. 2005, 2008). Nonetheless, the LOXL2 enzymatic function in particular has been shown to modulate the EMT-like phenotype in cancer cells (Barry-Hamilton et al. 2010).

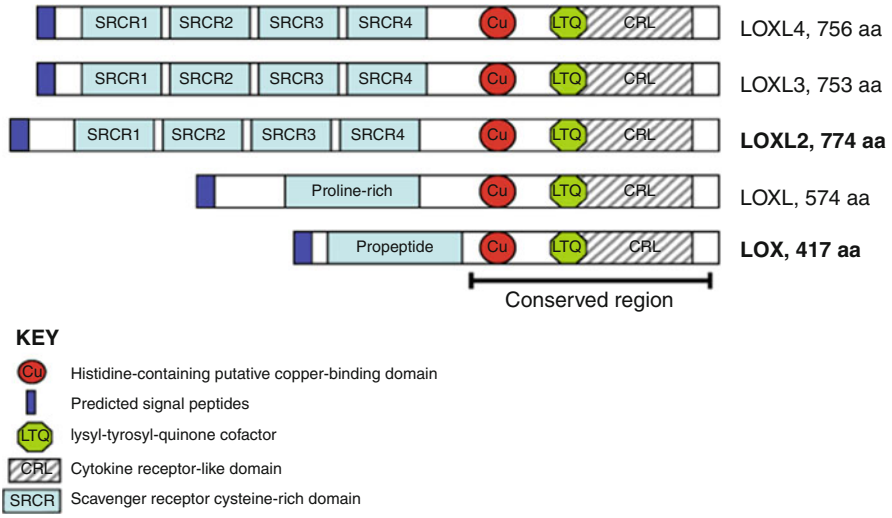


Fig. 3.2 The lysyl oxidase (*LOX*) family. The LOX family of proteins has a highly conserved C-terminal region that contains a copper-binding motif (depicted in *red*), a lysyl-tyrosyl-quinone (*LTQ*) cofactor (depicted in *green*), and a cytokine receptor-like domain (*shaded*). The N-terminal region is highly variable. LOXL2, 3, and 4 all have four scavenger receptor cysteine-rich regions (*SRCR*s; *light blue*), which is replaced by a proline-rich domain in LOXL (also known as *LOXL1*) and a propeptide region in LOX. *Purple boxes* depict predicted signal peptides

3.4.2 Hypoxic Regulation of Invasion

While EMT may strongly influence invasion, there are also other factors contributing to tumor cell invasion. As mentioned above, hypoxia-induced proteins such as LOX and LOXL2 also have extracellular roles that facilitate cancer cell invasion. LOX cross-links collagens, which increases matrix stiffness, thereby activating integrins that enhance cell-to-matrix adhesion, invasion, proliferation, and malignant transformation (Barker et al. 2012). The enzymatic function of hypoxia-induced LOX increases focal adhesion kinase activity, and proto-oncogene tyrosine-protein kinase Src activity, thereby mediating cell migration/invasion and metastasis in various cancer types (Erler et al. 2006; Baker et al. 2011, 2012). LOX also plays a role in the formation of premetastatic niches, which will be discussed in detail later.

Like LOX, both intracellular and secreted LOXL2 have been shown to be involved in focal adhesion kinase /Src activation in various types of cancers, mediating the invasive/migratory properties and thus metastasis of these cells (Peng et al. 2009; Moreno-Bueno et al. 2011). In addition, the enzymatic function of LOXL2 was involved in the invasion and metastasis of cancer cells through tissue remodeling, during which it regulated the expression and activity of tissue inhibitor of metalloproteinase-1 and MMP-9 (Barker et al. 2011).

Mobilized tumor cells first have to overcome the BM before they can successfully move through the ECM to intravasate into the circulatory system. The BM serves as a physical barrier between the cancer cells and the interstitial ECM and comprises collagen IV, entactin, laminin, glycoproteins, and proteoglycans. It is not normally permeable to cells, but tumor cells can alter their cell surface receptors, such as integrins, to allow contact with BM components and invade through this layer (Nicolson 1989; Liotta and Stetler-Stevenson 1991). Cancer cells also secrete enzymes to degrade the BM to allow easier penetration, such as cathepsin D, urokinase-type plasminogen-activator receptor, and MMP-2. These proteins all are upregulated by hypoxia. Hypoxia also induces fibronectin production, which facilitates cell motility through activation of integrins on the surface of tumor cells (Krishnamachary et al. 2003).

Another HIF-1 target, and thus one regulated by hypoxia, is the *MET* proto-oncogene (Pennacchietti et al. 2003). MET is a receptor tyrosine kinase that also interacts with integrins to activate downstream signaling events, leading to increased invasion/metastasis. Tumor cells overexpress *MET* under hypoxia, responding to the MET-ligand hepatocyte growth factor (HGF); CAFs have been known to secrete HGF under hypoxia (Ide et al. 2006). In this context hypoxia increases tumor cell motility both intrinsically (through *MET* overexpression on tumor cells) and externally (through HGF produced by CAFs). The tumor-secreted cytokine, autocrine motility factor, is another HIF-1 target that can also be induced by vascular endothelial growth factor (VEGF), another well-known hypoxia-induced growth factor. The autocrine motility factor enhances tumor cell proliferation and migration, and can act in either an autocrine or paracrine manner (Funasaka and Raz 2007).

3.4.3 Intravasation

Intravasation is the entry of tumor cells into the circulatory system, and it is most likely different to the exit of tumor cells from the circulation into distant metastatic sites (extravasation). Intravasation occurs at the tumor blood vessels, which often are malformed and irregular, thus allowing relatively easy access of tumor cells into the circulation. Extravasation, on the other hand, occurs at a distant organ site where the blood vessels are usually well formed and mature and thus present a tougher barrier for tumor cells to pass through. Nonetheless, tumor cells may still remodel the vessels to allow entry by secreting HIF-1α-regulated metalloproteinases MMP1 and MMP2 (Shyu et al. 2007), which have been shown to be synergistic mediators of vascular permeability and intravasation (Gupta et al. 2007). The MMP inhibitory protein RECK is also implicated in intravasation through HIF-1 upregulation of microRNA miR-372/373 (Loayza-Puch et al. 2010). Such observations open the possibility that other ECM proteins, such as LOXL2, could also play a role in the intravasation stage of metastasis; however, further investigations are required to elucidate the mechanisms of intravasation.

Hypoxia-induced VEGF, although often recognized as a angiogenic factor (detailed below), can also facilitate microvascular permeability and increase interstitial fluid pressure, thereby increasing the chances of intravasation by cancer cells (Sullivan and Graham 2007). It is interesting that the EMT-inducing and hypoxia-regulated transcription factor Twist, mentioned in the previous section, also has been found to increase the ability of tumor cells to intravasate (Yang et al. 2004), highlighting the complexity of the metastatic process.

3.5 The Influence of Hypoxia on Stromal Cells to Promote Tumor Cell Invasion

The tumor stroma consists of a noncellular component (the ECM), as described above, and a cellular component comprising a diverse range of nontumor "normal" cell types (Fig. 3.3). These stromal cells include fibroblasts, endothelial cells, perivascular cells, and inflammatory cells, and these all have been shown to play a significant role in cancer progression by mediating angiogenesis, desmoplasia, lymphangiogenesis, and inflammation (Finger and Giaccia 2010). In particular, CAFs and TAMs have gained much attention for their roles in promoting metastasis.

CAFs are myofibroblast-like cells that promote tumor growth by inducing desmoplastic reactive stroma around tumor cells (Kalluri and Zeisberg 2006). CAFs can induce tumor-promoting inflammation, enhance vascularization of primary tumor growth, and recruit immune cells that promote tumor progression. It also has been shown that normal fibroblasts can be educated by carcinoma cells to become proinflammatory and thus promote tumor progression (Erez et al. 2010). It has recently been demonstrated that hypoxia alone is sufficient to induce degradation of caveolin-1, a hallmark of CAFs that promotes higher tumor aggressiveness in patients with breast cancer, which can predict lymph node metastasis and chemoresistance. Moreover, the loss of stromal caveolin-1 also protects adjacent cancer cells against apoptosis and autophagy (Martinez-Outschoorn et al. 2010).

TAMs are recruited to areas of hypoxia, and their presence correlates with poor outcome in cancer patients (Bingle et al. 2002; Murdoch et al. 2004). It has been shown that TAMs form clusters within the primary tumors; these clusters are correlated with angiogenesis and elevated VEGF levels (Goede et al. 1999; Salvesen and Akslen 1999); however, this remains in dispute because other researchers did not find correlation between microvessel density and accumulation of macrophages in invasive carcinomas (Davidson et al. 1999). Host cells such as TAMs have been demonstrated to directly assist tumor cell intravasation without the need for local angiogenesis (Wyckoff et al. 2007), and it has been shown that TAMs accumulate in hypoxic areas because of hypoxia-induced secretion of macrophage chemoattractants such as endothelin 2 and VEGF by both tumor and stromal cells (Murdoch et al. 2004). It is intriguing that hypoxia can induce changes in the expression of a range of genes in normal macrophages (Table 3.3), including a range of proangiogenic factors (see the section "Hypoxia and Angiogenesis").

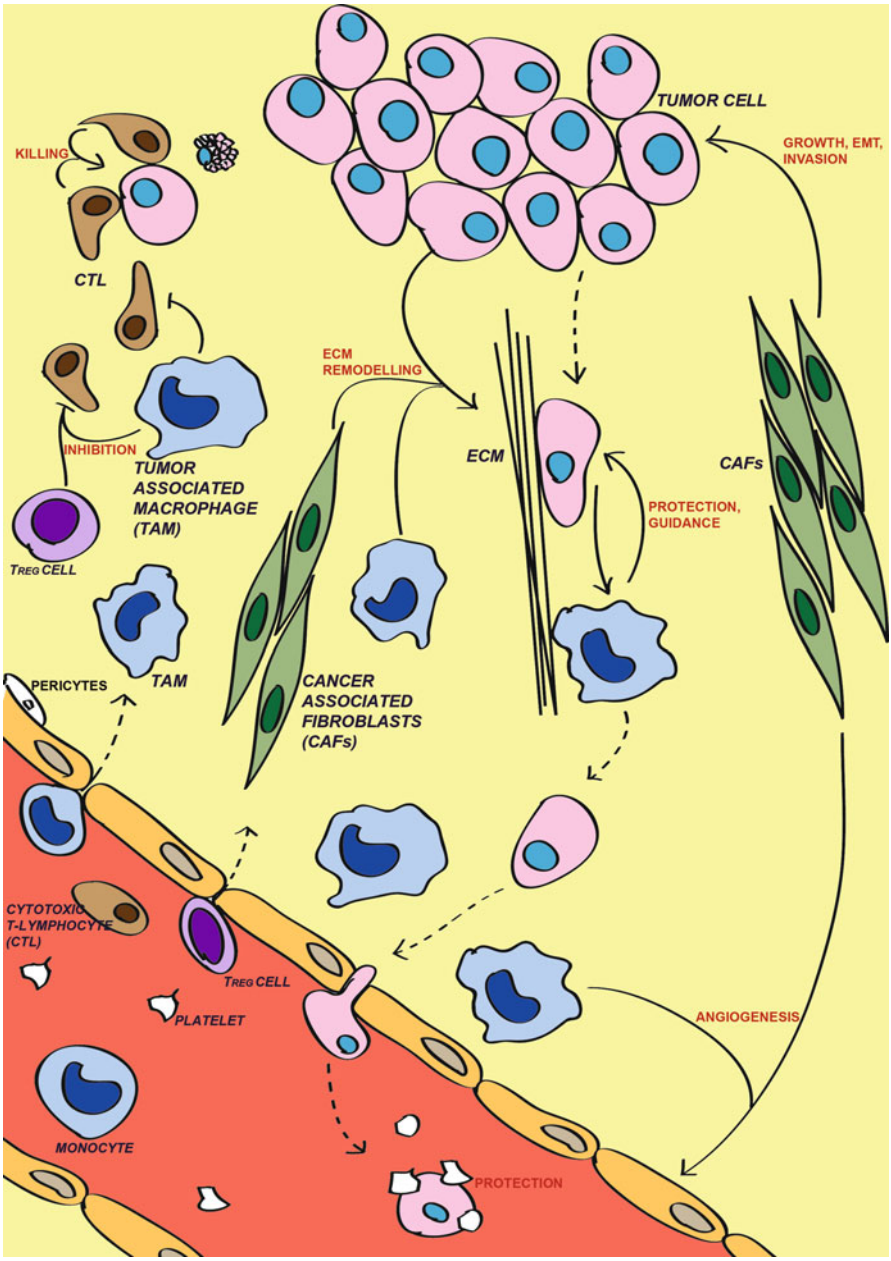


Fig. 3.3 The various stromal cells involved in tumor cell invasion. Host-derived stromal cells present in the tumor stroma can be involved in the invasion process of tumor cells and become cancer-associated stromal cells. For example, monocytes develop into tumor-associated macrophages (*TAMs*), and fibroblasts become cancer-associated fibroblasts (*CAFs*)

Table 3.3 Gene expression changes induced by hypoxia in normal macrophages

Factor	Function	Up-/downregulation
Glucose transporter-1	Survival	Up
Vascular endothelial growth factor	Proangiogenic	Up
Fibroblast growth factor-2	Proangiogenic	Up
PIGF	Proangiogenic	Up
Cyclooxygenase-2	Proangiogenic Prostanoid synthesis	Up
Leptin	Proangiogenic	Up
Platelet-derived growth factor-β	Proangiogenic	Up
Hepatocyte growth factor	Proangiogenic	Up
Fibronectin	Proangiogenic	Up
Angiopoietin-1	Proangiogenic	Up
Matrix metalloproteinase-1, -7	Proangiogenic Prometastatic	Up
Tissue factor	Proangiogenic Promigratory Prothrombotic	Up
Inducible nitric oxide synthase	Proangiogenic, proinflammatory	Up
Tumor necrosis factor-α	Proangiogenic Proinflammatory Cytotoxic	Up
IL-1	Proinflammatory	Up
Prostaglandin E_2	Immunosuppressive	Up
IL-10	Immunosuppressive	Up
CCL-2, -3	Chemokine	Down, up
C-X-C chemokine receptor type 4	Proangiogenic	Up
Interferon-γ	Immunostimulatory	Up
CXCL-8	Proangiogenic Chemokine	Up
CXCL-1	Chemokine	Up
NMB-R	Unknown	Up
CCR-5	Chemokine receptor	Down
CD80	Antigen presentation	Down
Robo4	Slit receptor Anti-angiogenic	Up
MIF	Antimigratory Prometastatic	Up
Very-low-density lipoprotein receptor	Proatherosclerotic	Up
ORP150	Proatherosclerotic	Up
IL-6	Proinflammatory	Up
Arginase	Proinflammatory	Up

IL interleukin

Adapted from Murdoch C, Muthana M, Lewis CE. Hypoxia regulates macrophage functions in inflammation. J Immunol 2005;175(10):6257–6263

3.6 Survival in Circulation

Tumor cells that have successfully entered the circulation (CTCs) can now travel to most organs in the body, provided that they first survive the harsh environment of the circulatory system. In particular, CTCs are vulnerable to anoikis, a form of apoptosis induced by a loss of adhesion that was first described by Frisch and Francis in 1994. CTCs also are subjected to the sheer forces exerted by blood flow as well as attacks from host immune cells (Gupta and Massague 2006). CTCs can increase their chance of survival by binding to platelets or lymphocytes for protection (Fidler and Bucana 1977; Gasic 1984; Nash et al. 2002) or by forming emboli by binding to coagulation factors such as thrombin, fibrinogen, and fibrin (Zhan et al. 2004). These emboli, also called heterotypic clumps because of the population of different cells, have a higher metastatic potential than aggregates of tumor cells alone (i.e., homotypic clumps) presumably because of the presence of host cells, and thus they are better shielded from immune cells. Nonetheless, the formation of aggregates, be it homotypic or heterotypic, probably also confers resistance to apoptosis by anoikis. From a diagnostic point of view, it has been demonstrated that the detection of CTCs in blood circulation has prognostic value in many carcinomas, suggesting a new noninvasive strategy of determining treatments for cancer patients (Gorges and Pantel 2013).

Hypoxia may seem to do little to assist CTCs because hypoxia is not present in circulating blood. However, the duration between intravasation and extravasation may only be a few hours, as demonstrated by in vivo videomicroscopy (Chambers et al. 1995); thus the hypoxia-induced response that occurs in the invasive cells in the primary tumor may act long enough to affect the survival and extravasation of CTCs, allowing them to successfully metastasize. It has been reported that hypoxia confers resistance to anoikis through suppression of α5 integrin, a sensitizer toward anoikis (Rohwer et al. 2008). Hypoxia also induces suppression of *Bim* and *Bmf*, thus inhibiting anoikis (Whelan et al. 2010). Another potential mediator is the hypoxia-responsive factor TrkB, which is a suppressor of anoikis (Martens et al. 2007).

3.7 Homing (Extravasation) and Metastatic Colonization

The first step for CTCs to successfully colonize a secondary site (and thus become DTCs) is to stop circulating and exit the blood vessel (extravasation) (Fig. 3.4). CTCs lodge in the capillary beds at secondary sites and may either extravasate and invade the foreign parenchyma as single cells or proliferate intraluminally and eventually rupture the wall of the microvessels (due to the size of the metastatic lesion), allowing the CTCs to enter the surrounding tissue (Al-Mehdi et al. 2000; Wong et al. 2002). Individual CTCs may become arrested mechanically because of their large size in comparison to the capillary lumen (Naumov et al. 1999; Ito et al. 2001) or through direct interaction with the surface molecules of endothelial cells (Nicolson 1988; Arap et al. 1998; Pasqualini et al. 2000). These processes may be facilitated by the endothelial cell P- and E-selectins (Mannori et al. 1997; Kim et al. 1998) as well as integrins and CD44 on the tumor cells (Birch et al. 1991; Ruoslahti 1994;

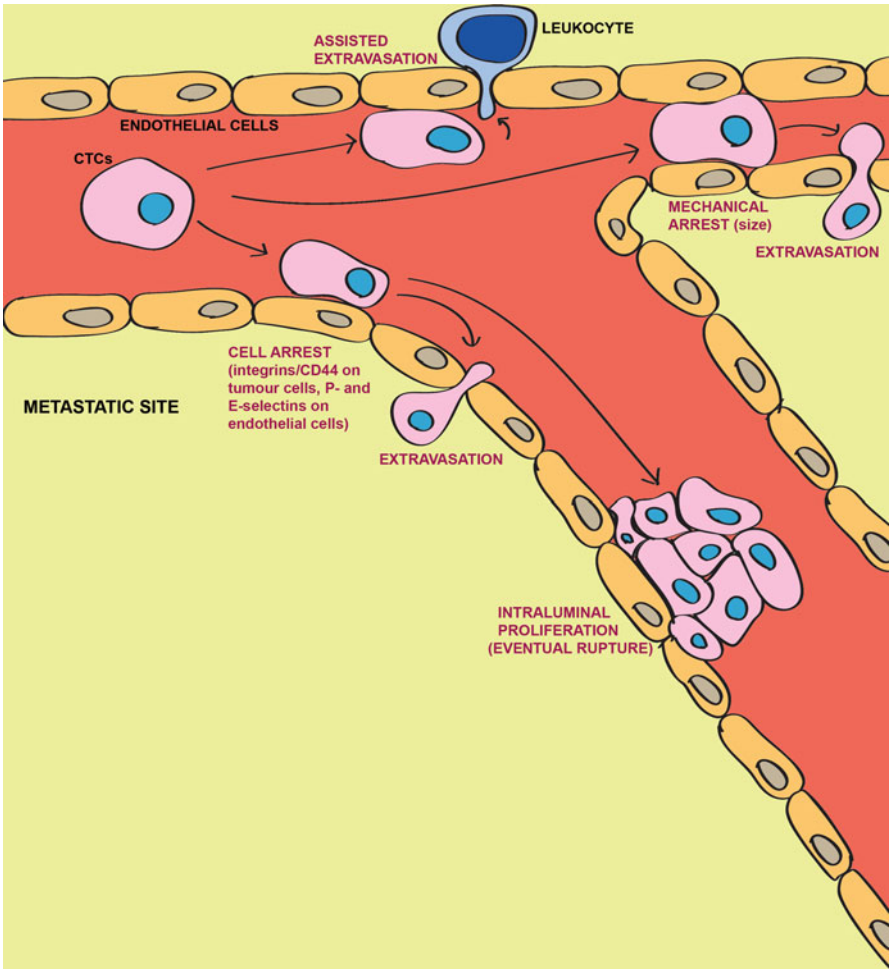


Fig. 3.4 The colonization process of circulating tumor cells. As a first step toward colonization of a distant site, the circulating tumor cells need to stop and undergo extravasation to leave the blood vessels. This can be achieved by various mechanisms: cell arrest extravasation, assisted extravasation, mechanical arrest extravasation, or intraluminal proliferation followed by rupture and spilling into the secondary site. Other cell types may be involved in this process (Adapted from http://www.nature.com/nrc/journal/v7/n10/pdf/nrc2229.pdf Figure 1)

Friedrichs et al. 1995; Wang et al. 2004). Because endothelial cells are constantly shed from blood vessels, in some cases CTCs may interact with the exposed BM directly through integrins such as α3β1 (Weiss et al. 1988; el-Sabban and Pauli 1994; Wang et al. 2004). This type of arrest can be enhanced by platelets aggregating with the CTCs, and ECM components such as fibronectin and laminin can also enhance tumor cell arrest (Terranova et al. 1984). Indeed, host cells such as leukocytes may also be recruited following CTCs arrest and lead the extravasation process, with the CTCs following them (Wood 1958; Sahai 2007).

3.7.1 Hypoxia and Extravasation

As mentioned earlier, the time between intravasation and extravasation may be only a few hours; thus the effects of hypoxia on CTCs may be maintained until after extravasation has occurred. This is supported by the observation that transient hypoxic treatment of tumor cells in vitro before intravenous injection into mice can increase colonization (Young et al. 1988) and that acute hypoxic treatment of the primary tumor (during tumor growth tumor-bearing mice were subjected to low oxygen conditions for 10 min 12 times a day) increases spontaneous metastasis (Cairns et al. 2001). Despite the fact that both intravasation and extravasation involve crossing the blood barrier, they are two different processes: tumor-associated blood vessels are usually highly permeable, while normal tissue vasculature at the metastatic site has a higher integrity and thus acts as a more effective barrier. Nevertheless, factors that contribute to intravasation may also be involved in extravasation. One such example is VEGF; it was shown that direct inhibition of VEGF can suppress extravasation and metastasis to the lungs in breast cancer (Lee et al. 2003).

3.7.2 Hypoxia and the Selection of Metastatic Sites

It has long been established that each type of cancer has favored metastatic sites, and this may be due to physical attributions of the various organs. One such attribution could be the structural differences of the capillaries in the organs, such as the sinusoid capillaries in bone marrow. This type of capillary has only a single layer of endothelial cells and no supporting structures, thus allowing easy trafficking of hematopoietic cells in and out of the bone marrow. This in turn presents an easy route along which cancer cells can extravasate, which may explain why bone marrow is the favored target organ for the metastases of a wide range of cancers (Alix-Panabieres et al. 2008). Another determining factor is the pattern of circulation within the body. For example, colorectal cancers have a strong preference for metastasizing to the liver (Schluter et al. 2006). In this case the blood circulation drains from the colon directly into the liver, bringing with it an extremely high number of CTCs (Chaffer and Weinberg 2011); thus even if the probability of a colorectal cancer cell being able to colonize the liver microenvironment is low, liver metastasis will still occur because of the sheer number of colorectal CTCs entering the liver.

The process of homing described above is likely to be a passive process; however, it is apparent that cancer cells actively seek out preferred organs to which they can metastasize, as the anatomical distribution of the metastases do not conform to the blood circulation pattern alone or the type of capillaries present. In fact, the idea was first put forward by Stephen Paget in 1989 as the "seed and soil" hypothesis: he proposed that metastatic spread is a combined result of properties intrinsic of the "seed," that is, cancer cells, and the properties of the secondary site – the "soil" – such that the compatibility of the tumor cells with the microenvironment is a predominant determinant of successful metastasis.

One such intrinsic property of cancer cells is the hypoxia-induced expression of the C-X-C chemokine receptor type-4 (CXCR4) (Murdoch 2000). CXCR4 allows CTCs to home in to tissues expressing high levels of the CXCR4-specific ligand stromal cell-derived factor-1 (SDF-1, also known as CXCL12) and has been shown to be important in various cancers such as renal cell carcinoma (Staller et al. 2003), ovarian cancer (Scotton et al. 2001, 2002), breast cancer (Lu et al. 2010), lung cancer (Liu et al. 2006), and neuroblastoma (Geminder et al. 2001).

Hypoxia has recently been demonstrated to affect the gene signatures of lung- and bone-specific metastases using different mechanisms. Hypoxia-induced angiogenesis genes are associated with lung metastasis but not bone metastasis, and hypoxia enhances a significant number of lung metastasis gene signatures, whereas only a few bone metastasis genes, such as the previously mentioned CXCR4 and dual specificity protein phosphatase 1, are induced by hypoxia (Lu et al. 2010).

Bone metastasis can be classified into two types: osteoblastic and osteolytic (Mundy 2002; Teicher and Fricker 2010). Osteoblastic metastases are commonly found in prostate cancer, whereas osteolytic metastases are commonly found in breast cancer and multiple myeloma. Regardless of the type of bone metastasis, osteoclast proliferation/activation and bone hypertrophy are commonly observed (Halvorson et al. 2006). Several known hypoxia-regulated proteins have been shown to drive osteolytic bone metastases. Connective tissue growth factor (Higgins et al. 2004) is involved in osteoclastogenesis and bone resorption, liberating tumor-promoting factors from the bone matrix (Kang et al. 2003; Nozawa et al. 2009). These tumor-promoting factors include bone morphogenic proteins and transforming growth factor-β, which also are upregulated by hypoxia signaling (Falanga et al. 1991; Maegdefrau et al. 2009). Hypoxia-induced osteopontin interacts with osteoclasts that express αvβ3, thus promoting bone metastasis (Engleman et al. 1997). The cytokines interleukin-6 and -8 are upregulated by hypoxia and have multiple functions that could promote bone metastasis: they induce angiogenesis, migration, and osteolysis (Bendre et al. 2005; Ara and Declerck 2010). VEGF also attracts VEGF receptor–positive tumor cells to the metastatic site.

It is widely accepted that hypoxia probably regulates various organ-specific metastases in different ways; however, it has been shown in animal models that inhibiting HIF-1α significantly reduced metastases to both lungs and bones, highlighting the importance of HIF-1α as a potential therapeutic target for multiple organotypic metastases (Lu et al. 2010).

3.7.3 Hypoxia and the Premetastatic Niche

In recent years the concept of the premetastatic niche has become increasing important, whereby factors secreted by tumor cells stimulate the preparation of distant sites of future metastasis, recruiting clusters of host bone marrow–derived cells (BMDCs) to home in and modify the microenvironment, thereby preparing the secondary sites to aid cancer cell colonization and growth (Psaila et al. 2006; Peinado

et al. 2011). The presence of these premetastatic niches greatly enhances tumor cell colonization and growth at the secondary site, and can influence the route of metastatic spread (Kaplan et al. 2006).

It was first demonstrated in 2002 that MMP-9 is induced in premetastatic lung endothelial cells by distant primary tumors through VEGF receptor 1 and is involved in lung-specific metastasis (Hiratsuka et al. 2002). The term *premetastatic niche*, however, was not coined until 2005, when it was demonstrated that host BDMCs expressing VEGF receptor 1 travel to premetastatic sites and form cellular clusters before tumor cells arrive, creating a permissive niche for incoming tumor cells. These events were shown to be influenced by factors secreted by the tumor cells. Exciting research showed that by introducing secreted factors of different tumors with distinct metastatic preferences, one could transform the metastatic profile and redirect organ colonization, indicating that premetastatic niches also guide CTCs to specific organs (Kaplan et al. 2005). It has since been shown that inflammatory chemoattractants affect both the primary tumor invasion and recruitment of myeloid cells to the lungs in the formation of the premetastatic niche (Hiratsuka et al. 2006).

Hypoxia also plays an important role in the formation of premetastatic niches. Hypoxic tumor cells secrete the aforementioned HIF-1 target LOX, which then accumulates at the premetastatic sites. The presence of LOX is essential for the recruitment of $CD11b^+$ myeloid cells to the premetastatic sites through matrix remodeling, allowing the $CD11b^+$ cells adhere to the ECM and secrete MMP-2. MMP-2 in turn cleaves collagens and in mouse models of breast cancer enhances further recruitment of BMDCs and invasion of CTCs (Erler et al. 2009). Furthermore, HIF-1 has been shown to be critical in the formation of the premetastatic niche in a breast cancer model through the induction of various members of the LOX family, including LOX, LOXL2, and LOX-like 4 (LOXL4). These LOX family members catalyze cross-linking of collagens at the site of premetastatic niche, promoting BDMC recruitment and thus enhancing lung colonization. It is interesting that each LOX family member is involved in the formation of the premetastatic niche of different subsets of breast cancer, highlighting the complexity of the cellular and molecular effects of LOX, LOXL2, and LOXL4, as well as the highly heterogeneous nature of responses to hypoxia (Wong et al. 2011). Of note, we recently showed that LOX mediates collagen cross-linking in normal lungs and livers in response to fibrotic signals, that this modified matrix is responsible for fibrosis-enhanced metastasis, and that altering collagen cross-linking alone was sufficient to significantly increase tumor cell proliferation (Cox et al. 2013). These findings suggest a key role for matrix remodeling mediated by hypoxia-regulated proteins at metastatic sites in enhancing metastasis. Exciting research showed that the HIF-1 inhibitors digoxin and acriflavine block the formation of premetastatic niches in breast cancer metastasis by inhibiting the hypoxia-induced expression of the LOX family members (Wong et al. 2012). In addition, inhibition of LOXL2 enzymatic activity modifies the tumor microenvironment by reducing the secretion of growth factors that are instrumental in invasion and metastasis by the cancer cells (Barry-Hamilton et al. 2010). This presents a therapeutic angle whereby breast cancer patients with high levels of HIF1 may benefit from inhibitors against HIF1 or against the LOX family.

3.7.4 Hypoxia and Secondary Tumor Growth

Metastatic tumor cells that have successfully disseminated into the metastatic site (i.e., DTCs) may become dormant as micrometastases for long periods of time before they can colonize efficiently and become macrometastases (Morris et al. 1994; Chambers et al. 1995; Pantel and Alix-Panabieres 2010). This dormancy may be due to the DTCs entering quiescence, because cell proliferation and cell death is balanced as a result of immune surveillance, or because of the lack of vascular support. It has been demonstrated in mice that the presence of hypoxia in a primary tumor is correlated with metastatic tumor growth (Buchler et al. 2004). Hypoxia-responsive genes such as *GPR56*, *KISS1*, and *CD82* (*KAI1*) can prevent DTCs from proliferating at the secondary sites (Horak et al. 2008; Nguyen et al. 2009). The transition of micrometastases to macrometastases requires vessel co-option or new blood vessel formation (angiogenesis) (Moserle et al. 2009; Kienast et al. 2010), and hypoxia is known to induce angiogenesis (Fraisl et al. 2009). Another hypoxia-inducible transcription regulator inhibitor of DNA-binding 1 (Id-1) can be upregulated by recruited BDMCs to promote progression of micro- to macrometastases (Gao et al. 2008). It is interesting that VEGF can also be involved in the progress of micro- to macrometastases by promoting vascularisation (see the next section).

3.8 Hypoxia and Angiogenesis

Angiogenesis is defined as the formation of new blood vessels. The process of angiogenic sprouting involves several steps and is regulated by the balance between angiogenic and anti-angiogenic factors present in the tissue. Angiogenesis is a critical step in cancer progression because it limits primary and metastatic tumor growth. It provides nutrients and oxygen to promote tumor growth and provides escape routes for cancer cells, allowing them to metastasize further.

Hypoxia induces the secretion of proangiogenic factors such as VEGF, angiopoietin 2, platelet-derived growth factor, fibroblast growth factor (Hanahan and Folkman 1996; Ruan et al. 2009) and decreases angiogenic inhibitors such as thrombospondin – all through HIF-1 (Laderoute et al. 2000). Of these, VEGF is characterized best. Tumor cells secrete VEGF, which stimulates endothelial cells and recruits endothelial progenitor cells. VEGF also stimulates outgrowth of pericytes and increases vascular permeability, as mentioned earlier (Senger et al. 1983; Leung et al. 1989). A mouse model of liver metastasis demonstrated that when micrometastases start to grow and become hypoxic, hepatic stellate cells are recruited to the hypoxic sites and secrete VEGF, which then recruits endothelial cells and promotes formation of the vasculature, thereby enabling macrometastases to develop (Olaso et al. 2003). The presence of the secondary vasculature can provide a route through which further metastases can occur. Of note, we recently showed that LOX regulates VEGF expression through activation of PDGFRβ,

resulting in *Akt* activation, which then upregulates the VEGF messenger RNA and protein (Baker et al. 2012). This demonstrates the involvement of ECM proteins such as LOX in angiogenesis.

The recruitment of circulating bone marrow–derived endothelial cells also increases angiogenesis (Nolan et al. 2007). In particular, the hypoxia-regulated SDF-1/CXCR4 pathway involved in the homing of CTCs is heavily involved in angiogenesis, as CXCR4 is also expressed by hematopoietic and endothelial cells, making this signaling pathway an attractive therapeutic target (Petit et al. 2007).

Angiogenesis is clearly important in enabling tumor progression at both primary and metastatic sites, and therefore it is understandable that much focus has been put on anti-angiogenic therapy in treating cancer. VEGFR inhibitors have been approved for treatment of patients with late-stage metastatic colorectal cancer, metastatic breast cancer, renal cell carcinoma, non-small-cell lung cancer, hepatocellular carcinoma, glioblastoma multiforme, medullary thyroid, and gastrointestinal stromal tumors (Crawford and Ferrara 2009), but they have generally yielded disappointing results. However, anti-angiogenic therapies also produce a paradox: inhibiting angiogenesis may cause hypoxia in the tumor, which in turn may promote metastasis, as described in this chapter. In preclinical models, VEGF receptor inhibitors such as sunitinib have been shown to have opposite effects on metastasis, depending on the type of cancer and treatment (Osusky et al. 2004; Ebos et al. 2009; Paez-Ribes et al. 2009; Zhang et al. 2009), underscoring the complexity of targeting angiogenesis. This was demonstrated in a recent study in which two murine carcinoma models (mammary carcinoma and renal adenocarcinoma) treated with various doses of sunitinib showed varying effects of the drug – not just on the metastases but also on myeloid cell recruitment and survival (Welti et al. 2012). Nonetheless, large-scale clinical studies have reported that blocking VEGF does not aggravate the clinical outcome of patients with advanced-stage metastatic disease; in fact it prolongs progression-free survival or overall survival of these patients (De Bock et al. 2011). However, rapid tumor regrowth has been reported in some cancer patients after anti-angiogenic therapy is withdrawn (Burstein et al. 2008). This suggests that anti-angiogenic therapy may not be optimal on its own, and coupling it with therapy targeting HIF1 may actually be of benefit to eliminate potential hypoxic promotion of cancer progression caused by inhibition of angiogenesis.

3.9 Conclusions

Metastasis is a highly complex, multistep process and is responsible for the majority of deaths among cancer patient. Hypoxia is correlated with treatment failure, metastasis, and poor patient survival. However, there is increasing evidence demonstrating that hypoxia potently influences every step of the metastatic process, driving cancer progression. Thus, targeting hypoxia may successfully reduce or even prevent cancer metastasis. It should be remembered that the heterogeneity of hypoxia within the tumor indicates complex hypoxia-mediated metastasis, and most studies

to date have focused on individual proteins and thus lack perspective on how these are interconnected. This highlights the need for further research into hypoxia-regulated metastasis, taking a more systems-biology approach to investigate the dynamics of the molecular networks involved and determine how best to target these. Nonetheless, it is clear that targeting hypoxia may be beneficial as an adjuvant to existing cancer therapies.

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