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Hypoxia and the extracellular matrix: drivers of tumour metastasis

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Abstract

Of the deaths attributed to cancer, 90% are due to metastasis, and treatments that prevent or cure metastasis remain elusive. Emerging data indicate that hypoxia and the extracellular matrix (ECM) might have crucial roles in metastasis. During tumour evolution, changes in the composition and the overall content of the ECM reflect both its biophysical and biological properties and these strongly influence tumour and stromal cell properties, such as proliferation and motility. Originally thought of as independent contributors to metastatic spread, recent studies have established a direct link between hypoxia and the composition and the organization of the ECM, which suggests a new model in which multiple microenvironmental signals might converge to synergistically influence metastatic outcome.

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Rapid cancer cell proliferation, combined with structural and functional abnormalities in tumour blood vessels, results in regions within solid tumours that have reduced oxygen availability[1]. Oxygen availability decreases as the distance from the nearest blood vessel increases. Intratumoural hypoxia is associated with disorganized vascular networks with intercapillary distances that are greater than the diffusion distance of oxygen (which is ~100–200 μm, depending on the local oxygen concentration in the blood and the rates of oxygen consumption)[2]. The direct measurement of the tumour partial pressure of oxygen (PO_2) using Eppendorf microelectrodes (which remains the gold standard for determining oxygen levels) has revealed that patients whose primary tumours are poorly oxygenated (those with PO_2 <10 mmHg) have an increased risk of metastasis and mortality[1,3,4]. The best understood mechanism of how cancer cells adapt to a hypoxic environment is through the transcriptional activity of hypoxia-inducible factors (hypoxia-inducible factor 1 (HIF1) and HIF2; see BOX 1)[5]. The role of hypoxia and HIFs in reprogramming cancer cells by regulating the expression of multiple genes involved in angiogenesis, by regulating the metabolism of glucose and by regulating cancer cell invasion and metastasis has been extensively reviewed elsewhere[6–10]. Recent reviews also highlight the importance of hypoxia in recruiting the stromal cell components of the tumour microenvironment[11,12]. In this Opinion article, we focus on how hypoxia affects extracellular matrix (ECM) deposition, remodelling and degradation, which might potentiate cancer metastasis. Central to this emerging paradigm are three crucial findings: the ECM is a dynamic structure that influences tumour progression[13–18]; multiple cell types, including cancer cells, contribute to ECM production[19–23]; and the remodelled ECM within regions of intratumoural hypoxia could be a pathway rather than an obstacle for cancer metastasis[24–27].

The ECM and cancer

The ECM is composed of approximately 300 proteins that regulate tissue homeostasis, organ development, inflammation and disease[19]. The major constituents of the ECM are fibrous proteins (such as collagens, elastins, fibronectins and laminins) and proteoglycans (such as chondroitin sulphate, heparan sulphate, keratan sulphate and hyaluronic acid) that are locally secreted and assembled into an organized mesh, which forms the structural framework for most tissues[28]. Molecular approaches aiming to correlate clinical outcomes with specific gene expression patterns within the primary tumour have highlighted genes that encode tumour-associated ECM components[19,29–34]. An increased expression of genes encoding proteins that mediate ECM remodelling has been associated with increased mortality in patients with breast, lung and gastric cancers[35,36]. These studies corroborate histological findings that show an excessive ECM deposition (also termed fibrosis) within solid tumours[37–43].

The most well-recognized ECM alteration that occurs in the tumour tissue is increased collagen deposition[44–53]. Collagens are the most abundant ECM components, constituting up to 90% of the ECM and 30% of the total protein in humans, and they provide the structural integrity and the tensile strength of human tissues and organs[54]. In the context of cancer biology, collagens regulate the physical and the biochemical properties of the tumour microenvironment, which modulate cancer cell polarity, migration and signalling[17,55–58]. Collagen I deposition and cancer metastasis have been causally linked using mice

engineered to express a collagenase-resistant α1 chain of type I collagen (*Col1a1*^{tm1jae} mice)[59]. *Col1a1*^{tm1jae} mice were crossed with mouse mammary tumour virus promoter-driven polyoma middle T antigen (*MMTV-PyMT*) transgenic mice to model increased type I collagen deposition during the progression of human breast cancer[59–61]. *Col1a1*^{tm1jae}*; MMTV-PyMT* bitransgenic mice had a threefold increase in the incidence of tumour formation and metastasis compared with their wild-type littermates[60]. Furthermore, histological studies of human breast carcinomas have shown that fibrosis is localized to hypoxic regions within tumours and correlates with immunostaining of the HIF1 target gene product carbonic anhydrase IX (CAIX)[51,52]. Highly fibrotic tumours also have the highest CAIX immune reactivity, which can independently predict patient relapse rate and shorter disease-free survival[51,53]. In this Opinion article, we discuss emerging data that has provided experimental evidence linking the mechanisms of hypoxia-induced collagen deposition and remodelling to those of invasion and metastasis.

Tumour ECM synthesis and degradation

The current view of tumour fibrosis suggests that recruited and resident fibroblasts and myofibroblasts within the primary tumour are mediators of tumour fibrosis. These cells are activated by proteins that are secreted by cancer cells, most notably by transforming growth factor-β (TGFβ), which stimulates the synthesis of ECM proteins and the remodelling of the ECM by proteases produced by cancer-associated fibroblasts[62]. Fibroblasts that are isolated from the site of a healing wound or from fibrotic tissues secrete higher levels of normal ECM constituents and proliferate more than their normal counterparts that are isolated from healthy organs, which is an explanation for the increase in matrix deposition that occurs within a tumour[63]. Although it is well accepted that invasive carcinoma is often associated with increased ECM deposition in tumours[64], there is also evidence for an increased deposition of ECM in hypoxic tumour regions. Recent studies have uncovered mechanisms of tumour fibrosis that specifically occur under hypoxic conditions and that involve not only fibroblasts but also other cell types, including cancer cells[20,21,65–68].

Hypoxia induces increased collagen gene expression

Hypoxia and HIF1 have been implicated in renal, liver and adipose tissue fibrosis[69–71]. Dermal, cardiac and renal fibroblasts cultured under hypoxic conditions show increased type I procollagen α1 mRNA levels[72–74]. Furthermore, increased levels of type I, II and IV procollagen mRNA are present in the peripheral lung parenchyma and pulmonary artery of rats that have been exposed to hypoxia[75]. However, studies that describe the regulation of collagen gene expression in hypoxic cancer cells *in vitro* and during cancer progression *in vivo* are lacking. By contrast, the dramatic effect of hypoxia on the post-translational modification of collagen is a matter of considerable investigation, as described below.

HIF1 regulates the expression of intracellular collagen-modifying enzymes

Collagen biogenesis originates with gene transcription and is followed by the translation of mRNA into procollagen (pro-α-chains) (FIG. 1). At least 28 collagen subtypes, which are encoded by 42 genes that generate 42 distinct pro-α-chains, have been identified in vertebrates[76]. Within the endoplasmic reticulum, the pro-α-chains undergo multiple post-

translational modifications, which include the hydroxylation of proline and lysine residues, followed by the glycosylation of hydroxylysine residues[76]. The modification of proline to 4-hydroxyproline is essential for the thermal stability of the collagen triple helix[77]. Procollagen α-chains that are not hydroxylated are improperly folded, which leads to proteolytic degradation and to reduced collagen deposition[76,78]. Three isoforms of the prolyl 4-hydroxylase α-subunit (P4HA) have been identified (P4HA isoform 1 (P4HA1), P4HA2 and P4HA3) that form A_2B_2 tetramers with P4HB, which results in the generation of P4H1 (from P4HA1), P4H2 (from P4HA2) and P4H3 (from P4HA3) holoenzymes[79,80]. Three procollagen-lysine 2-oxyglutarate 5-dioxygenase genes (*PLOD1*, *PLOD2* and *PLOD3*) encode enzymes that mediate collagen lysine hydroxylation. Collagen crosslinks that are derived from hydroxylated lysine residues compared with non-hydroxylated lysine residues have increased stability, which leads to increased tissue stiffness[81]. Thus, stiff tissues, such as bones, cartilage and tendons, contain a higher percentage of hydroxylated lysine residues in collagen compared with soft tissues, such as the skin[81].

HIF1 regulates the expression of P4HA1, P4HA2, PLOD1 and PLOD2 in cancer cells, fibroblasts, chondrocytes and endothelial cells[20,21,67,82–86]. Abrogating the expression of HIF1α, P4HA1 or P4HA2 through the stable transfection of cells with short hairpin RNA (shRNA) vectors inhibits collagen deposition from both breast cancer cells and fibroblasts *in vitro*[21,82]. Reducing the levels of HIF1α, P4HA1 or P4HA2 *in vivo* results in decreased fibrosis and decreased tissue stiffness in orthotopic tumours that are formed by the injection of human breast cancer cells into the mammary fat pads of immunodeficient mice[21,68]. Decreasing the levels of HIF2α expression in breast cancer cells had no effect[21]. Importantly, P4HA1 or P4HA2 knockdown inhibited the spontaneous metastasis of breast cancer cells to the lungs and to the lymph nodes of mice by reducing the formation of collagen fibres, which are required for cancer cell adhesion, spreading and invasion[21,68]. In contrast to P4HA1 and P4HA2, the depletion of PLOD2 in breast cancer cells did not suppress collagen deposition *in vitro* or *in vivo*, but reduced tumour stiffness by reducing fibrillar collagen content[20]. PLOD2 knockdown also significantly impaired the invasion of cancer cells into the adjacent normal tissue of the mouse mammary fat pad, reduced the number of circulating tumour cells and prevented the spontaneous metastasis of breast cancer cells to the lungs and to the lymph nodes of mice[20]. In murine models of sarcoma, abrogating HIF1-dependent PLOD2 expression disrupted collagen modification, cell migration, and pulmonary metastasis[67]. Taken together, the studies described above indicate that hypoxia might regulate ECM deposition by multiple cell types within the tumour microenvironment[20,21,67,68,82,83,86]. In addition to the marked effects of collagen prolyl and lysyl hydroxylase expression in experimental mouse models of metastasis, *P4HA1*, *P4HA2* and *PLOD2* expression have also been suggested as biomarkers for human cancer progression in several independent studies (BOX 2).

HIFs regulate extracellular collagen-modifying enzymes

Following enzymatic modification of type I collagen by hydroxylation, two α1(I)-chains and one α2(I)-chain associate to form a triple helix that is secreted into the extracellular space (FIG. 1). Collagen peptidases cleave the carboxy- and amino-terminal peptides, and type I collagen fibrils form spontaneously, are covalently crosslinked on hydroxylysine and lysine

residues and form structurally stable collagen I fibres[76,87]. Fibrillar collagens, such as type I collagen, establish the interstitial matrix and contribute to tissue stiffness with extensive post-translational modifications that increase tensile strength[54]. Non-fibrillar collagens, such as type IV collagen, constitute a key component of the basement membrane, which is a compact sheet-like structure that functions as a barrier to separate tumour cells from the adjacent stroma[88].

Collagen crosslinking is extracellularly initiated by the lysyl oxidase (LOX) family of secreted enzymes that oxidatively deaminate lysine or hydroxylysine collagen residues[87]. Three LOX enzymes — LOX, LOX-like protein 2 (LOXL2) and LOXL4 — are important hypoxia-induced and HIF-regulated target gene products that are involved in collagen crosslinking and tumour fibrosis[65,89–93]. In addition to collagen crosslinking within the primary tumour, secreted LOX has been shown to localize within the lungs and to remodel existing collagen to establish a premetastatic niche containing bone marrow-derived cells (BMDCs), which facilitates colonization of the niche by cancer cells in murine models of breast cancer[90–91]. LOX family members are upregulated to varying levels and in different combinations in human breast cancers[93]. Similarly, breast cancer cell lines show different patterns of LOX family member expression in response to hypoxia, but in each case the expression is HIF dependent[89]. Consideration of the specific LOX family members that are induced by hypoxia is therefore essential to prevent collagen remodelling, BMDC recruitment and metastasis in the lungs of tumour-bearing mice[89,90]. The pharmacological inhibition of LOX by β-aminopropionitrile (βAPN) has been reported to inhibit metastasis in experimental mouse models; however, βAPN might not inhibit the activity of all LOX family members[93], which suggests that HIFs or pan-LOX inhibitors could represent broader targets than currently available drugs or antibodies that target only a subset of LOX and LOXL proteins.

HIF1 and HIF2 can regulate ECM degradation

In addition to collagen deposition, collagen degradation also contributes to ECM remodelling and is mediated by several families of proteinases that have been suggested to promote cancer cell invasion; for example, the matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that are divided into several subgroups (collagenases, gelatinases, stromelysins and cell membrane-bound MMPs) with different substrate specificities. Hypoxia is associated with an increase in the expression and the activity of type IV collagen-degrading enzymes (MMP2 and MMP9) *in vitro*[94–96]. *MMP2* and *MMP9* are upregulated by hypoxia in breast and colon cancer cells via a HIF1-dependent mechanism[94–96], whereas membrane-bound membrane-type 1 MMP (MT1-MMP; also known as MMP14) is upregulated in a HIF2-dependent manner[94,97]. In addition to collagen degradation by MMPs, hypoxic cancer cells also show increased proteolytic activity as a result of HIF-dependent increases in their expression of urokinase plasminogen activator surface receptor[98,99] (PLAUR). PLAUR promotes cell invasion by altering the interactions between integrins and the ECM. When PLAUR expression levels are depleted by the expression of shRNAs, cells with reduced levels of PLAUR are incapable of intravasation[100]. Thus, HIFs activate a transcriptional programme that results in the degradation of the basement membrane while simultaneously increasing the *de novo*

synthesis of fibrillar collagens to function as a physical pathway for tumour invasion (FIG. 2).

Growth factors and ECM deposition

Tumours have long been described as ‘wounds that won’t heal’ (REF. 101). Similarly, hypoxia is known to have a role in both normal and pathological wound healing. In normal cutaneous wounds, HIF1 is important for appropriate angiogenic responses, for mobilization of circulating angiogenic cells, such as endothelial precursor cells and mesenchymal stem cells (MSCs), and for normal wound contraction[102]. Partial reduction of HIF1α expression is consequently sufficient to impair wound healing[103]. During wound healing, angiogenesis and ECM deposition occur in parallel[104]; therefore, it is not surprising that some of the same factors that stimulate angiogenesis also promote fibrosis. Hypoxia-induced angiogenic growth factor production has been well established[105]. HIF1 has been shown to bind to a *cis*-acting hypoxia-response element in the genes that encode vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF1; also known as CXCL12), angiopoietin 2 (ANG2), platelet-derived growth factor B (PDGFB), placental growth factor (PGF), connective tissue growth factor (CTGF) and stem cell factor (SCF)[106–114] and can also indirectly promote fibroblast growth factor 2 (FGF2)[115] production in a variety of cell types (TABLE 1).

Although well-known for their influence on tumour angiogenesis, many of these growth factors also contribute to fibrosis[116– 118], potentially by attracting fibroblasts to the primary tumour and/or by activating resident fibroblasts. Experimental evidence indicates that the recruitment of fibroblasts or myofibroblasts to sites of pathological fibrosis is driven by hypoxia[119–121]. Similarly, hypoxia increases the recruitment of bone marrow-derived MSCs in murine models of breast cancer, which results in increased lymphatic and vascular metastasis[112,122]; for example, VEGF — which is released by hypoxic cancer cells but more often by endothelial cells, fibroblasts and inflammatory cells — has been implicated in fibrosis because of its role in stromal cell activation and because it leads to the production of an ECM that is rich in fibronectin and type I collagen[63]. VEGF also induces microvascular permeability, which in turn mediates an influx of fibroblasts, inflammatory cells and endothelial cells to the primary tumour[123].

Hypoxia and macrophage recruitment

Hypoxia-induced growth factor secretion in the primary tumour also promotes the accumulation of macrophages, which rapidly respond to the hypoxic microenvironment by altering their gene expression patterns[124–126]. The importance of hypoxia in stimulating macrophage infiltration during wound healing has been shown in heterozygous HIF1α-deficient mice, which show considerable delays in myeloid cell infiltration[127]. Recent studies highlight a potential mechanism of macrophage recruitment into hypoxic regions involving the release of semaphorin 3A by hypoxic cancer cells, which functions as an attractant for macrophages that express neuropilin 1 (NRP1)[128]. Once in the region of hypoxia, macrophages stimulate fibrosis by producing growth factors such as TGFα, TGFβ1, VEGF, FGF, PDGF, tumour necrosis factor-α (TNFα), interleukin-1 (IL-1) and

IL-8, which can attract additional macrophages and mesenchymal cells, such as fibroblasts and endothelial cells, and can further activate stromal cells[129]. Macrophages also directly promote the process of cancer cell intravasation into nearby blood vessels[130]. In addition, they contribute to ECM turnover by secreting MMPs, which suggests that the identification of a specific macrophage subpopulation and/or soluble mediator that preferentially promotes or degrades the ECM might be an important determinant of the extent of fibrosis within a tumour[129]. Taken together, the studies described above suggest that hypoxic signalling engages multiple cell types that contribute to ECM remodelling within the tumour microenvironment (FIG. 3).

Physical properties of tumour ECM

The physical properties of the tumour ECM refer to its stiffness, topography, porosity and solubility[131]. The physical properties of the tumour-associated ECM are not only fundamentally different from the ECM of normal tissues but are also continuously remodelled[17,132], which reflects the dynamic changes that occur in the tumour microenvironment, including changes in oxygen availability.

Tumour Stiffness

Tumour stroma is typically stiffer than normal stroma; for example, breast tumours can be ten times stiffer than normal breast tissue[57,133] and expression of collagen-modifying enzymes, such as P4HA1, P4HA2, PLOD2 and LOX, that can be induced by hypoxia promote tumour stiffness[20,21,58,65,66,82]. Stiffening of the ECM causes a reciprocal increase in the traction forces that are exerted by a cell[134,135]. Intracellular contraction in response to ECM stiffening results in an increase in the stiffness of the actin cytoskeleton and an increase in cell migration[57,58,136–138]. Increased tumour stiffness might regulate tumour progression in several ways; for example, increasing matrix stiffness increases RHO-generated cytoskeletal tension to promote focal adhesion assembly and to increase growth factor-dependent ERK activation[123,135]. Moreover, matrix stiffness facilitates integrin clustering, leading to the activation of focal adhesion kinase 1 (FAK1), which in turn activates the MAP/ERK kinase (MEK; also known as MAP2K)–ERK pathway and leads to increased cell survival, migration, invasion and proliferation[57,138,139]. Depletion of FAK1 in mouse tumour models inhibits local invasion and metastasis, which indicates that FAK1 activation might be an important mediator of stiffness-induced tumour metastasis[140–142]. Interestingly, matrix stiffening can lead to a feed-forward signalling mechanism that further increases matrix stiffening; for example, YAP1 is required for matrix stiffening by cancer-associated fibroblasts during tumour progression[143]. Conversely, stiff matrices and the contractile actin cytoskeleton further increase YAP1 activation[143].

Tumour topography

In addition to the changes in matrix stiffness that occur during tumour progression, the topography of the ECM is also highly dynamic. For example, invasive breast cancers often contain type I collagen fibres that are oriented perpendicular to the tumour margin at the invasive front, in contrast to the non-oriented fibrils that are often seen in less aggressive breast cancers[25,26,144,145]. Straightened and aligned collagen fibres are found at sites of

breast cancer invasion — a histological pattern that is termed tumour-associated collagen signature 3 (REF. 146), which is associated with decreased patient survival[25]. Similarly to breast cancer, in early melanomas, collagen is localized to the periphery of the tumour[144,145,147]. By contrast, metastatic melanomas have a less compact ECM structure with no barrier between the cancer cells and the adjacent normal tissue. Highly aligned collagen fibres within a tumour might not just be predictive of the metastatic potency of the tumour but may also be causative given the finding that cancer cells preferentially invade along straightened and aligned collagen bundles[24,26,148].

The ECM isolated from wild-type fibroblasts that have been exposed to hypoxia is more highly aligned than the ECM deposited by fibroblasts that have been cultured under ambient conditions, and collagen fibre alignment under hypoxic conditions is abrogated in fibroblasts transfected with shRNA against HIF1α[82]. Breast cancer cells that have been plated on ECM produced by hypoxic cells are highly aligned and migrate with directional persistence along ECM fibres, in contrast to cells that have been plated on ECM produced by non-hypoxic cells, which migrate in a random manner[21]. Similarly, in an orthotopic mouse model of breast cancer, aligned ECM fibres are present in the perinecrotic (hypoxic) region of control tumours; by contrast, tumours that are derived from breast cancer cells expressing shRNA against HIF1α have a disorganized ECM comprised of almost no fibrillar collagen[21].

One potential mechanism of collagen alignment in hypoxia might involve the activity of the small GTPase RHOA, which interacts with RHO-associated protein kinase 1 (ROCK1) to mediate myosin II phosphorylation, resulting in cell contraction. RHOA-mediated ROCK1 activity is required for caveolin 1-induced cell contraction, which enables cancer cells to align with and potentially to migrate along the pre-existing collagen matrix *in vitro*[149]. Experiments *in vivo* also indicate that caveolin 1-dependent regulation of RHOA is required for fibroblasts to produce an aligned matrix[149]. In renal clear cell carcinoma, caveolin 1 is a direct transcriptional target of HIF1 and HIF2 (REF. 150). Moreover, hypoxia coordinately regulates the expression of RHOA and ROCK1 through HIF1- and HIF2-dependent transcription in breast cancer cells, which results in increased cell-induced matrix contraction[151]. An alternative or an additional mechanism of collagen alignment could involve LOX expression. Second harmonic generation (SHG) imaging of mammary glands that have been preconditioned with LOX-expressing fibroblasts shows that they contain more linearized collagen than the mammary glands of control mice[58]. Taken together, these data suggest a model in which hypoxic cells can generate and organize an aligned ECM through multiple mechanisms (FIG. 2).

Biochemical properties of the ECM

The structure of the ECM influences the stability and the bioavailability of growth factors and cytokines[14], many of which are generated under hypoxic conditions (TABLE 1). The balance between ECM-mediated confinement or distribution of growth factors and their concentration will determine their availability to cell surface receptors and consequently will regulate intracellular signalling[14]. A highly aligned ECM might more readily establish a chemoattractive gradient that potentiates hypoxic signalling. Conversely, a dense collagen network could function as a sink for growth factors and thereby could reduce their rate of

diffusion. Future studies to determine the ECM arrangement and composition that supports the optimal distribution of growth factors to mediate metastasis might lead to a better understanding of how the ECM influences cancer cell motility and dissemination.

Potential therapeutic interventions

HIF inhibitors

Increased expression of HIF1α and HIF2α has been observed in a broad range of human cancers and has been associated (in most but not all cases) with a poor prognosis[152–154], which suggests that use of HIF inhibitors has the potential to improve patient survival not only by blocking ECM deposition but also by blocking dozens of other HIF target genes that encode proteins involved in cell survival, angiogenesis, metabolic reprogramming, immortalization, epithelial-to-mesenchymal transition (EMT), stem cell maintenance, resistance to radiation and chemotherapy, invasion and metastasis[155]. Although considerable work has been done to characterize the role of HIFs in experimental cancers with regards to tumour incidence and growth[152], the direct requirement for HIFs in metastasis has only recently been shown in both orthotopic models and autochthonous breast tumour models[156,157]. Conditional knockout models of HIFs have also aided our understanding of how the hypoxic tumour environment affects different cell types to drive cancer progression; for example, loss of either HIF1α or HIF2α in mouse vascular endothelial cells has been shown to reduce tumour growth because of impaired angiogenesis[158,159]. Conversely, haplodeficiency of prolyl hydroxylase domain-containing protein 2 (PHD2; also known as EGLN1) increases the HIF-driven upregulation of expression of VEGF receptor 1 (VEGFR1) in endothelial cells and decreases intratumoural hypoxia, resulting in decreased HIF1α expression in cancer cells, which reduces pulmonary metastasis[160,161]. The studies described above suggest that clinical trials are warranted for HIF inhibitors that show efficacy in preclinical models. It will also be crucial to determine how the activity of each HIFα subunit is affected by the potential inhibitor, given the reported functional differences between HIF1α and HIF2α[162,163].

Two inhibitors of HIF1α accumulation that have shown anticancer effects in preclinical models are the topoisomerase I inhibitor topotecan[151] and the cardiac glycoside digoxin[90,106,157]. In addition to reducing the expression of many HIF target genes, treatment of tumour-bearing mice with digoxin reduces tumour fibrosis, as well as lymph node and lung metastasis[90,106,157]. A Phase II clinical trial for digoxin is currently being carried out for men with recurrent prostate cancer (ClinicalTrials.gov, number: NCT01162135). In addition, a pilot clinical trial of topotecan (ClinicalTrials.gov, number: NCT00182676) in patients with advanced cancer and HIF1α overexpression shown on tumour biopsy was recently reported in which HIF1α protein levels were undetectable in the post-treatment biopsy samples from four of seven patients who were studied, and decreased tumour blood flow was observed in 70% of patients by contrast-enhanced dynamic magnetic resonance imaging[164]. Neither trial assessed treatment-induced changes to the tumour ECM. However, preclinical use of the HIF1α inhibitor PX-478 or overexpression of a dominant-negative HIF1α mutant showed that the increased fibrotic response identified in fat pads from mice that were fed a high-fat diet could be effectively prevented by treatment with PX-478. The

preclinical effectiveness of PX-478 has previously been established in tumour models where treatment reduces tumour growth[165] and it will be interesting to determine the effect of PX-478 on tumour fibrosis.

Targeting fibrosis

Blocking collagen hydroxylases or lysyl hydroxylases might also provide a strategy to reduce tumour fibrosis. P4Hs have been regarded as attractive targets for the pharmacological inhibition of collagen accumulation in fibrotic diseases and severe scarring. P4Hs belong to a superfamily of dioxygenases that use oxygen and α-ketoglutarate (also known as 2-oxoglutarate) as substrates. P4Hs are competitively inhibited by α-ketoglutarate analogues, including *N*-oxalylglycine, pyridine 2,4-dicarboxylate and pyridine 2,5-dicarboxylate, coumalic acid and 3,4-ethyl dihydroxybenzoate (EDHB)[166]. As these agents are not selective for collagen hydroxylases, it is probable that they will also inhibit the HIF PHDs and will potentially promote HIF expression. Preclinical testing will have to be carried out to determine their potential usefulness in preventing metastasis. Minoxidil has been shown to decrease the expression of PLOD mRNAs and the activity of PLOD proteins and thereby to inhibit fibrosis[167]. In a mouse model of sarcoma, minoxidil treatment reduced tumour fibrosis and suppressed lung metastasis[67].

Studies that target LOX family members have focused on blocking the enzymatic activity of these proteins using competitive inhibitors such as βAPN[168] or using neutralizing antibodies, which abrogate lung and liver metastases in xenograft and transgenic mouse models[93]. D-penicillamine (DPEN), which is a LOXL2 inhibitor, was developed and used to treat rheumatoid arthritis and biliary cirrhosis but it does have some unintended side effects[169]. A more selective inhibitory monoclonal antibody (AB0023) against LOXL2 has been developed and was effective in reducing fibrosis in primary and metastatic xenografts as well as in liver and lung fibrosis models in mice[170]. The ECM of tumours from mice that had been treated with AB0023 showed a marked reduction in crosslinked collagen compared with results in mice that had been treated with the lysyl oxidase inhibitor βAPN[170]. Treatment with AB0023 also resulted in a marked reduction in the number of activated fibroblasts and endothelial cells and led to a decreased production of growth factors and cytokines[170]. The safety of the humanized version of AB0023, AB0024 (also known as simtuzumab) has been tested in Phase I dose escalation trials in patients with advanced solid tumours[171] (ClinicalTrials.gov, number: NCT01323933) and with idiopathic pulmonary fibrosis (ClinicalTrials.gov, number: NCT01362231). Enrolment for a Phase II clinical trial in patients with idiopathic pulmonary fibrosis (ClinicalTrials.gov, number: NCT01759511) has begun. Additional non-selective inhibitors of lysyl oxidases also include *p*-halobenzylamines, ethylenediamine and homocysteine thiolactone[93,172].

Conclusions

Although tumours from two different patients might have similar genetic alterations, these tumours will develop in different microenvironmental contexts[13], which suggests that hypoxia and the ECM are important in contributing to tumour heterogeneity, which might influence metastatic outcome. Hypoxic regions within the tumour microenvironment can

simultaneously relay signals to cancer cells and cells that have been recruited to the local environment directly (for example, by transcriptional reprogramming), through paracrine signalling events and, as highlighted in this Opinion article, by establishing a hypoxia-induced ECM that is fibrotic, stiff and aligned, which are all properties that promote metastatic dissemination[11,173]. Further studies are needed to investigate the mechanisms by which the hypoxia-induced ECM might have a role in dynamically maintaining and distributing growth factors that provide chemotactic signals to recruit cells to the primary tumour and that promote the intravasation of cancer cells for dissemination to distant organs. Advances in imaging techniques, such as intravital microscopy, have the potential to shed light on this issue and might direct our research to appropriate targeting strategies that will be most beneficial to prevent metastasis[174].

It is also important to consider that the collagen-modifying enzymes discussed in this Opinion article might have alternative roles in cancer progression that are not limited to fibrosis[93]. For example, LOX has a role in PDGF and insulin growth factor 1 (IGF1) signalling, but its precise mechanisms of action remain to be elucidated[175]. Additional regulators of the collagen hydroxylases and lysyl oxidases remain to be determined; for example, TGFβ1 has been shown to influence LOX expression[93]. Furthermore, the role of collagen in the regulation of ECM composition and assembly (and vice versa) is also unknown; for example, collagen I-containing fibrils do not form in the absence of fibronectin *in vivo* and fibronectin fibril assembly has a reciprocal requirement for collagen[176]. Whether fibronectin–procollagen interactions are established before the molecules are secreted is unknown and suggests that the complex regulation and dynamics of the ECM need to be carefully investigated in order to design strategies that target the ECM. Another important consideration will be the receptors that interact with the ECM molecules. A recent study regarding the fibrillar collagen receptor discoidin domain receptor 2 (DDR2) has shown that DDR2 is required for breast cancer cell invasion and migration *in vitro* and for metastasis *in vivo* by promoting the stabilization of SNAIL1 (REF. 177).

Future preclinical studies are warranted to identify new inhibitors and/or to identify optimal combinations of existing inhibitors that can block hypoxic changes to the ECM while maintaining the integrity of the ECM in healthy tissues. One major obstacle in the field of cancer therapeutics for metastasis is the definition of success. Many agents effectively target tumour growth but fail to prevent metastasis, which is the major cause of cancer mortality. For metastasis inhibitors to be tested in early phase clinical trials patients that do not already have metastatic disease will have to be included in order to have meaningful end points and to establish efficacy in metastasis prevention.

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Box 1

Regulation of HIFs

Hypoxia-inducible factors (HIFs) are transcription factors that function as heterodimers, which consist of an oxygen-regulated HIF1α (or HIF2α) subunit and a constitutively expressed HIF1β subunit[178,179]. HIFs bind to the consensus sequence 5′-RCGTG-3′ that is present within or near HIF-regulated genes[180]. HIF1α protein levels are regulated by oxygen-dependent prolyl hydroxylation, which is required for binding of the von Hippel–Lindau (VHL) tumour suppressor protein, leading to ubiquitylation and proteasomal degradation of HIF1α[181]. Hydroxylation of HIF1α residues Pro402 and Pro564 is catalysed by HIF prolyl hydroxylase domain-containing protein 1 (PHD1), PHD2 and PHD3 in a reaction that is dependent on the presence of cofactors, oxygen and α-ketoglutarate (also known as 2-oxoglutarate). Under low oxygen conditions, HIF1α hydroxylation, ubiquitylation and degradation are inhibited[153]. HIF2α, which shares 48% amino acid sequence identity with HIF1α, is also oxygen-regulated and binds to HIF1β to form HIF2, which activates the transcription of some, but not all, HIF target genes[182,183]. Many oncogenic alterations in cancers cells, including loss of function of VHL, PTEN and p53[184–186], as well as activation of the PI3K–AKT[187] pathway, also cause an increase in HIF activity. Data obtained from many recent studies that use a range of approaches have revealed unique roles for HIF1α and HIF2α in both normal and cancer cells[152].

Box 2

Collagen hydroxylases and cancer

Procollagen-lysine 2-oxyglutarate 5-dioxygenase 2 (*PLOD2*) was included among the genes shown to be upregulated in gene expression screens of cervical cancer[188], glioblastoma[189] and gastric cancer[190], and was 1 of 17 genes that predicted breast cancer metastasis to the brain[191]. Gene expression studies also revealed increased *PLOD2* mRNA expression in primary sarcoma samples from patients with metastatic compared to non-metastatic sarcomas[67]. Moreover, human osteosarcoma samples have two to three times more hydroxylysine content than normal bone collagen, which indicates that PLOD activity is increased in these patients[44]. Increased prolyl 4-hydroxylase α-subunit isoform 1 (*P4HA1*) expression was revealed by a meta-analysis that was used to identify genes that are upregulated across many different cancer types[192]. P4HA2 was determined to be a metastasis-associated protein in oral cavity squamous cell carcinoma using comparative tissue proteomics[193]. Increased *P4HA2* expression levels also discriminated papillary thyroid cancer from normal thyroid tissue[194]. Increased *P4HA1*, *P4HA2* or *PLOD2* mRNA expression is predictive of breast cancer patient survival; the predictive power is improved when the expression of all three genes is evaluated and determined to be greater than the median expression level[20,21].

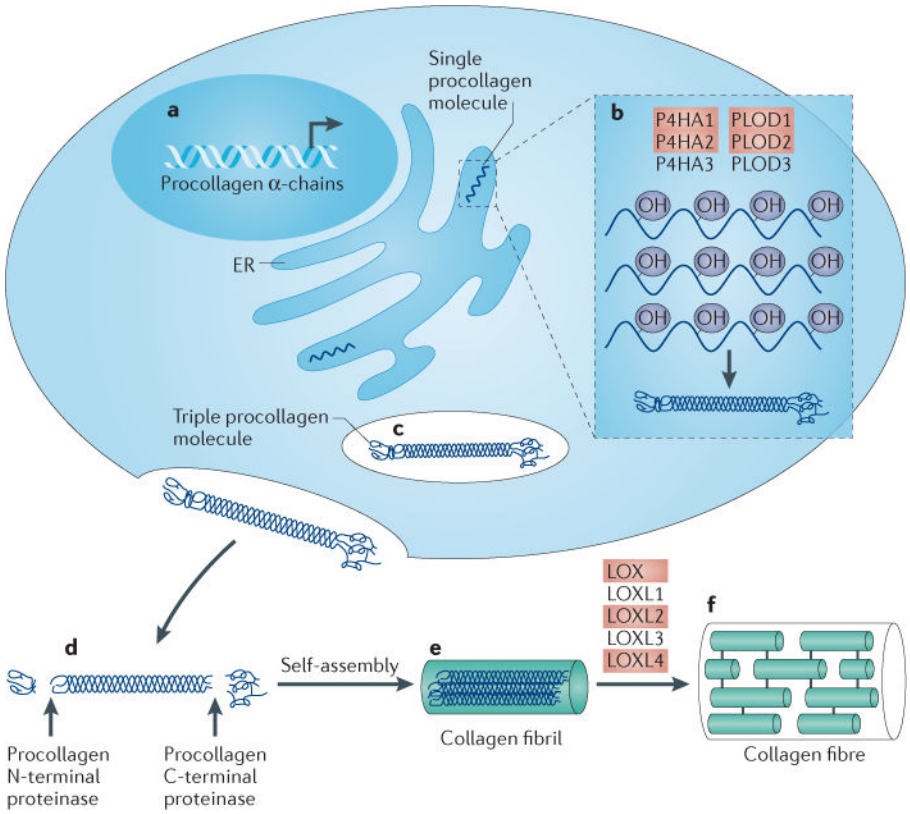

Figure 1. Biosynthesis of fibrillar collagens

The biosynthesis of type I collagen and other fibrillar collagens can be divided into intracellular (parts **a**–**c**) and extracellular (parts **d**–**f**) steps. The first intracellular step involves the synthesis of procollagen polypeptides from any of 42 distinct collagen gene transcripts (part **a**). Procollagens are post-translationally modified within the cisternae of the endoplasmic reticulum (ER) by prolyl 4-hydroxylase α-subunit isoform 1 (P4HA1), P4HA2 and P4HA3 and by procollagen-lysine 2-oxyglutarate 5-dioxygenase 1 (PLOD1), PLOD2 and PLOD3 lysyl hydroxylase enzymes (part **b**). Hydrolysine residues can be further modified to galactosyl hydroxylysine and to glucosylgalactosyl hydroxylysine by collagen galactosyltransferase and glucosyltransferase, respectively. The carboxyl termini of three properly hydroxylated procollagen molecules will associate and spontaneously propagate a procollagen triple helix from the carboxyl terminus to the amino terminus. The triple helical procollagen will be transported from the ER to the extracellular space via the Golgi (part **c**). Two metalloproteinases, a procollagen N-terminal proteinase and a procollagen C-terminal proteinase, cleave the non-helical termini (part **d**) and the mature collagen proteins spontaneously aggregate to form a collagen fibril (part **e**). The final step, collagen fibre formation, is initiated by collagen crosslinking, which is catalysed by lysyl oxidase (LOX) family members and occurs via the lysine aldehyde- or hydroxylysine aldehyde-initiated pathway (part **f**). The number and the proportion of the various crosslinks are tissue specific and are regulated by the steric relationship between localized collagen molecules, the type of collagens co-polymerized and the glycosylation and the hydroxylation of the participating amino acid residues. For example, lysine aldehyde-initiated crosslinks are found in soft connective tissue, in contrast to hydroxylysine aldehyde-initiated crosslinks, which are

found in stiff connective tissues. Many non-fibrillar collagens retain a non-collagenous N- or C-terminal, which prevents the spontaneous formation of collagen fibrils, and in these collagens cysteine crosslinks might be the only source of covalent intermolecular bonds. Enzymes highlighted in red are induced under hypoxic conditions. LOXL, LOX-like protein.

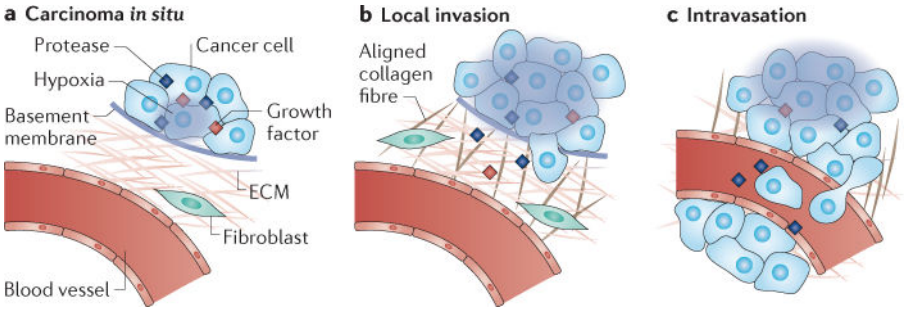

Figure 2. Hypoxia promotes ECM remodelling to facilitate metastasis

Extracellular matrix (ECM) remodelling is tightly controlled to maintain tissue integrity. Cancer cells and associated stromal cells that have been exposed to hypoxia are transcriptionally reprogrammed to produce: matrix metalloproteinases (MMPs) and other proteases, which degrade the basement membrane surrounding a tumour (part **a**); aligned collagen fibres within the interstitial matrix, which function as a highway for local invasion, intravasation and metastasis (part **b**); and growth factors, which might be retained in the fibrotic microenvironment and function as chemotactic signals that recruit and activate stromal cells to further promote cancer progression (part **c**).

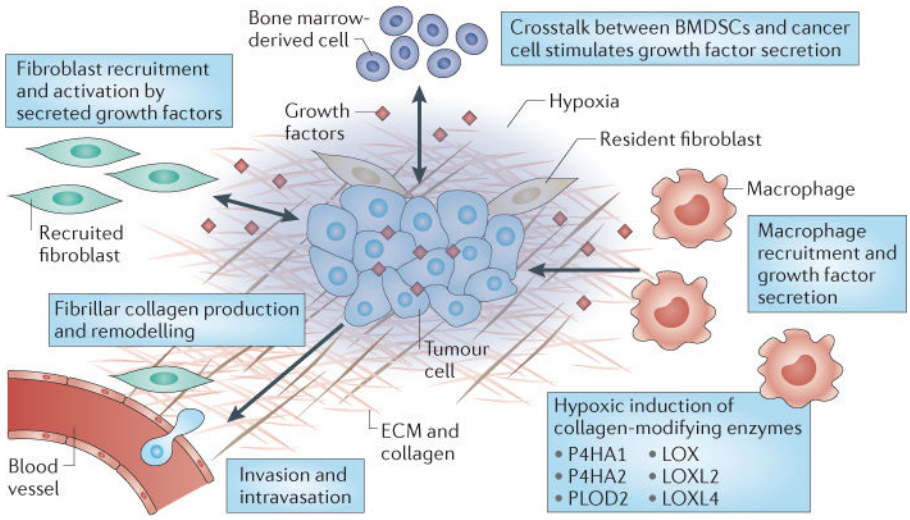

Figure 3. Hypoxia recruits and reprogrammes cells to produce fibrillar collagen

Hypoxia-induced and hypoxia-inducible factor (HIF)-regulated growth factor secretion by tumour cells promotes the recruitment of macrophages and fibroblasts to hypoxic regions of the primary tumour. Macrophages produce growth factors such as transforming growth factor β1 (TGFβ1) and platelet-derived growth factor (PDGF) that activate recruited and resident fibroblasts to stimulate collagen deposition. Hypoxic cancer cells also signal to mesenchymal stem cells, which might participate in collagen deposition. HIFs regulate the production of collagen-modifying enzymes, including prolyl 4-hydroxylase α-subunit isoform 1 (P4HA1), P4HA2, procollagen-lysine 2-oxyglutarate 5-dioxygenase 2 (PLOD2), lysyl oxidase (LOX), LOX-like protein 2 (LOXL2) and LOXL4 to facilitate the proper maturation of collagen fibres. Together, these signalling pathways promote the production of a fibrillar collagen network (that is produced by multiple cell types), which increases the ability of cancer cells to invade blood vessels. BMDSCs, bone marrow-derived stem cells; ECM, extracellular matrix.

Table 1

Factors induced by HIFs and their role in fibrosis

Factor induced by HIFs	Role in fibrosis
latelet-derived growth factor	Stimulates the replication, the survival and the migration of myofibroblasts[118]
Connective tissue growth factor	Promotes collagen deposition by myofibroblasts[195]
Fibroblast growth factor 2	Promotes the proliferation and the differentiation of endothelial cells, smooth muscle cells and fibroblasts, and stimulates collagen deposition[196]
Endothelin	Promotes fibroblast activation, proliferation and differentiation into myofibroblasts[197]
Angiotensin	Stimulates TGFβ production[198] and promotes collagen I and collagen III deposition[199]
Insulin growth factor 2	Increases connective tissue growth factor-stimulated collagen deposition[200]
CXC-chemokine ligand 2	Promotes fibrocyte recruitment[119]

HIFs, hypoxia-inducible factors; TGFβ, transforming growth factor-β.