Hif expression and the role of hypoxic microenvironments within primary tumours as protective sites driving cancer stem cell renewal and metastatic progression.

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Abstract

Hypoxic microenvironments frequently exist in many solid tumours with oxygen levels fluctuating temporally and spatially from normoxia to hypoxia. The response to hypoxia in human cells is mainly regulated by hypoxia-inducible factors or HIFs, a family of transcription factors which orchestrate signalling events leading to angiogenesis and tumorigenesis. Several events conspire together to lead to the stabilisation of HIF-α, commonly expressed in many cancer cell types. These events can result from low oxygen tensions occurring within the expanding tumour mass to produce hypoxic microenvironments or from mutations whereby the HIFs cause changes in expression of genes involved in several cellular functions. Hypoxia-mediated HIF-α regulation has gained significant prominence in tumour biology over recent years and the hypoxic microenvironments have been shown to facilitate and trigger major molecular and immunological processes necessary to drive the progression of tumours to malignancy. More recently, it has been realized that the hypoxic microenvironments also play significant roles in shielding tumour cells from immune attack by promoting immune suppression. In addition, the hypoxic microenvironment promotes many other oncogenic events such as the metabolic reconfiguration of tumour cells, neovascularization, epithelial to mesenchymal transition (EMT), and cancer stem cell renewal and accumulation. This article reviews the molecular mechanisms underlying tumour hypoxia and their pro-tumour contributions such as immune suppression, development of nascent and more permeable tumour vasculature, selective cancer stem cell renewal, accumulation, mobilisation and promotion of EMT leading to tumour cell metastasis.
Introduction:

Hypoxia, a condition of oxygen deprivation that compromises biological function, is a common phenomenon observed in many human pathologies including its growing role as an important factor in cancer [1] Tumour progression is normally sustained by the provision of a constant nutrient supply via its associated vasculature, as long as this can remain sufficient to meet the metabolic requirements of the growing tumour [2]. As most tumours proliferate rapidly, they commonly undergo periods of null, limited or even aberrant vasculature which meet the nutritional and oxygen requirements of the growing tumour [3]. As a survival mechanism, tissues exposed to oxygen deprivation release signalling factors leading to neovascularisation, often associated with subsequent tumour progression.

Hypoxia-inducible factors (HIFs) are a family of three transcriptional regulators that mediate the effects of key oxygen sensors in the cells. Thereby control the expression of genes that are major contributors to neovascularisation, including the signalling factors like vascular endothelial growth factors (VEGFs) [4] and erythropoietin (EPO) [5]. Besides these, HIFs also alter the expression of several genes encoding specific glycolytic protein isoforms (enzymes and transporters) [6, 7]. The HIF mediated modification in expression of glycolytic proteins causes a metabolic reprogramming, which helps to address the energy crises inside growing tumours enabling their to survive the state of hypoxia [8] and also confer resistance to chemo- and radiotherapy treatments [9]. However, the contribution of the HIFs to drug resistance differs depending on the cancer type and cells of origin [8, 10]. Thus, tumours showing increased HIF-α protein stabilisation often also exhibit higher levels of radio-resistance [11] whereas low HIF-α detectable protein levels are not predictive for radio-sensitivity. Experiments have shown significant variability in the levels of HIF-1α protein detected in human tumour cell lines, with high levels present even under normoxic conditions [12]. These variations directly correlate with the levels of expression of carbonic anhydrase CA-IX, a HIF-1α down steam target gene [13]. The variability in HIF-α protein levels may be a result of varying tumour dynamic properties due to cycling hypoxic episodes whereby the cells undergo repeated hypoxia and reoxygenation cycles [14].

HIF-α stabilisation shows a distinct correlation with pathophysiological phenomenon like tumour invasiveness and metastasis [15]. The present review will focus on factors contributing to HIF-1α stabilisation, and its multifaceted role in promoting pro oncogenic events, like epithelial-mesenchymal transition (EMT), metastasis [16],selective accumulation and maintenance of cancer stem cells and tumour survival by immune suppression.

HIFs and tumour hypoxia:

The HIFs are a highly conserved family throughout evolution. The HIF transcription factor when formed into its active state comprises a heterodimer of either one of three different α-subunits (HIF-1α, HIF-2α or HIF-3α) together with a β-subunit, HIF-1β. Unlike the HIF-1α subunit, which primarily takes part in the hypoxic response, HIF-1β, also called thearyl hydrocarbon nuclear translocator [ARNT], participates in cellular responses to environmental toxins including several exogenous ligands such as natural plant flavonoids, polyphenols and indoles, as well as synthetic polycyclic aromatic hydrocarbons and dioxin-like compounds [17]. The human HIF-1α and HIF-1β comprise 826 and 789 amino acids, respectively, and both proteins contain a basic helix-loop-helix domain involved in DNA binding, and Per/ARNT/Sim (PAS)
domain which enables the HIF-1α-β subunit dimerization [18]. The oxygen dependent domain (ODD) present on the HIF-1α protein makes it vulnerable to degradation under normoxia. Besides the ODD, HIF-1α also contains 2 domains required for transcriptional activation (TAD), the C-terminal activation domain (CTAD) and the N-terminal activation domain (NTAD) [19]. CTAD controls the regulation of HIF target genes whereas NTAD contributes to gene specific targeting [20] (Figure 1).

HIF-2α shares structural similarity to HIF-1α, but it shows tissue-specific expression, limited to the kidneys, small intestine, endothelium, lungs and heart [21]. Under hypoxic conditions, HIF-2α fails to undergo proteasomal degradation but forms dimers with HIF-1β to activate hypoxia response element (HRE)-mediated gene regulation [22]. The function of HIF-3α is not fully understood, but its splice variant is known to inhibit transcriptional activity of HIF-1α [20]. HIF-1α stabilisation and function inside cancer cells is represented schematically in Figure 2. Recently, bi-functional activity of the two HIF-1α transcriptional domains was demonstrated by Dyan et al [23], when they demonstrated that both C-and N-terminal transcriptional activation domains differentially contributed towards HIF-1α activity. Each of these domains was shown to have their own independent function in that they were hydroxylated by prolyl hydroxylase PHD2 and asparaginyl hydroxylase, respectively [23]. Reversal of the hypoxic effect was demonstrated by selectively inactivating HIF-1α using siRNA-mediated repression in cells undergoing hypoxia, resulting in a complete or partial shift in EMT markers back to the pre-hypoxic state, accompanied by a significant decrease in the hypoxia-induced migration and invasion of tumour cells [24].

Role of PHD, FIH and VHL in HIF regulation:

The prolyl hydroxylases (previously also known as EgLN) are a family of enzymes that includes 3 members, termed PHD1, 2 and 3 [25], each having a highly conserved gene structure [26]. Although all isoforms are known to catalyse hydroxylation reactions in vitro, each isoform has a different subcellular localization, substrate specificity, tissue expression and contribute differently to the regulation of HIFs [27, 28]. The PHD enzyme is part of an intrinsic oxygen sensing mechanism that under normoxic conditions prevents the stabilisation of the HIF-1α subunits and their ability to contribute to tumorigenesis. The PHDs act by promoting the physical interaction between VHL and HIFα, which targets HIF-α’s for ubiquitination and degradation by the proteasomal complex. For example, in human cells, the enzymatic function of PHD is to hydroxylate the proline residues P402 and P564 on HIF-1α to promote the VHL binding [22, 29]. A ten percent decrease in the oxygen concentration has demonstrated a rapid lowering in hydroxylation of proline residues on HIF-1α resulting in its nuclear stabilisation [30] (see Figure 3 for regulation of PHD activity as a function of the O2 concentration).

The PHD1 mRNA levels are highest in testes while PHD2/3 mRNA levels are highest in heart tissue [17]. Studies have indicated that upon reoxygenation, HIF-1α regulates the self-degradation of PHD2 and PHD3 [31]. This observation reinforces the importance of the PHD2/3 isoforms in the hypoxic response and in addition, mammalian PHD2 and 3 mRNAs are HIF-1α inducible, probably through the presence of HIF transcription sites in their promoters, whereas PHD1 is not induced by HIF-1α [32, 33]. It has been speculated that PHD2 and 3 are efficient regulators of HIF-α, even under hypoxic conditions. Under prolonged hypoxic conditions, increased PHD2/3 expression was found to be associated with a decrease in HIF-1/2α protein levels, while their mRNA levels showed no significant alteration [31]. Silencing experiments conducted in a range of different cell lines targeting the genes encoding the PHD enzymes, revealed that PHD1 and 3 had no effect on elevation of HIF-1α levels. On the contrary, silencing of PHD2 (EgLN1) resulted in an upregulation of HIF-1α expression [17]. SiRNA transfection
experiments demonstrated that PHD2 plays a pivotal role in hypoxic regulation [34] and showed that the PHD2 isoform is most important for HIF-1α down regulation in normoxia and mild hypoxia, promoting HIF-1α degradation via hydroxylation of the P564 residue [34]. PHD2, but not PHD1/3, is necessary for normal embryonic development as demonstrated by targeted gene disruption of PHD2 which resulted in embryonic lethality, whereas PHD1/3 double knock-out resulted in viable mouse embryos [34]. The results summarised here converge to emphasise that PHD2 is the most crucial component of HIF regulation and normal physiology compared to its counterparts. The role of PHD2 in different cell lines, its expression and regulation at the protein level and its correlation with clinicopathological parameters still has yet to be addressed and understood completely.

Factor inhibiting hypoxia 1 (FIH1), like PHD, is another oxygen sensor involved in the regulation of HIF-1α activity [23]. It indirectly affects HIF-1α levels by preventing the binding of the transcriptional co-activator p300/CBP, and inhibits HIF-1α by inactivating the C-terminal transactivation domain rather than affecting its level of expression [35]. The FIH1 gene encodes an asparaginyl hydroxylase which hydroxylates the Asn-803 residue in the C-terminal activation domain (CTAD) of the HIF-1α protein [36](Figure1). Hypoxic abrogation of asparaginyl hydroxylase activity results in the recruitment of a large transcription activating complex onto hypoxic response target genes, providing a second oxygen sensing mechanism participating in the hypoxia response pathway [37]. Recently, a null mutation introduced into the murine FIH gene revealed that it had no significant effects on HIF function. Instead, this FIH mutation caused a hypermetabolic phenotype whose modifications included a lower body weight as well as increased glucose and lipid metabolism [38]. Interestingly, this would make FIH a potential target for treatment of diseases based on hypermetabolic symptoms and obesity. However, little investigation has occurred into whether FIH in humans plays the same role in metabolism as that in the mouse model, and this is an area that requires further study.

Once HIF-1α subunits become hydroxylated by PHD, the VHL protein comes into play by recognising and binding to the hydroxylated HIF-1α, leading to ubiquination and proteasomal degradation, thereby keeping HIF-1α at low levels under normoxic conditions. Hence, VHL is a HIF-1α regulatory protein [39] and loss of function mutations in VHL result in a variety of tumours, including pheochromocytoma, although precisely how these tumours develop is still under intensive investigation. Many different mutations are responsible for VHL gene inactivation and several missense mutations have been linked to pedigree families with pheochromocytoma. VHL is a multipurpose adaptor involved in inter-protein interactions controlling diverse HIF-1α dependent functions. VHL gene loss of function or inactivating mutation is an early requirement in cancer development and can occur spontaneously [40], initiating pathological events due to the localisation of the gene product in the mitochondria, an organelle that contains angiogenic factors and enzymes required for HIF-1α regulation [41].

The VHL gene in humans encodes a 213 amino acid protein (pVHL) that has two domains with as yet unknown enzymatic function [42]. pVHL forms a multimeric complex with elonginB, C, Cul2 and Rbx1 proteins, known as the VBCR complex, that recognises hydroxylated HIF-1α or HIF-2α and targets them for proteolytic degradation (Figure1), [43, 44]. Disruption of the VHL/HIF-α interaction can lead to HIF-α stabilisation, followed by nuclear translocation and dimerization with HIF-ß, and the complex then switching on the angiogenic and tumourigenic signals [22, 45, 46] (Fig. 2). Knock-in of wtVHL genes into VHL-/- renal clear carcinoma cells suppressed their ability to form tumours in vivo [47] and, interestingly, studies have also shown that VHL is able to target non-hydroxylated HIF-1α for degradation during hypoxia [48].
Mitochondrial metabolites in tumour hypoxia and a key role for succinate as a PHD regulator

Pseudohypoxia is a phenomenon whereby HIF-1α becomes aberrantly stabilised, even under normoxic conditions [49]. It is often associated with the mutational inactivation of genes encoding the mitochondrial enzymes succinate dehydrogenase (SDH) and fumarate hydratase (FH), and is proposed to be the reason for the commonly associated mutations of these genes found in tumours such as pheochromocytoma and paraganglioma [50], as well as in clear cell renal carcinomas [51]. The mutations in the SDH, FH and isocitrate dehydrogenase (IDH) genes result in the accumulation of their respective substrates [52] increasing the levels of carboxylic acids, most significantly, succinate, fumarate and citrate, as well as pyruvate and lactate [53]. Several of these carboxylic acids can directly inhibit the activity of the PHDs [52, 54] (Figure 3). Consequently, in a similar fashion to the VHL mutation, the inhibition of PHDs by these metabolites results in prevention of HIF-α degradation, increasing its cellular levels [54] (see Figure 1). The high intracellular lactate levels found in cancer cells can induce increased HIF-1α stabilisation, but works indirectly via its conversion to pyruvate in a reaction catalysed by lactate dehydrogenase [55].

A build up in succinate levels accumulating in the cytosol inhibits PHD and thereby can trigger hypoxic signals, even under normoxia [55]. In the mitochondria, succinate couples the TCA cycle to mitochondrial respiration where succinate is used as a substrate of Complex II. However, it can also be transported out of the mitochondrial matrix to the cytosol and succinate is the downstream product of the oxidative decarboxylation of 2-OG, the one 2-oxo acid metabolite required for activating the PHDs. In the cytosol, succinate acts as a stable regulator, high concentrations of which result in inhibition of PHD (Figure 3), leading to HIF-1α stabilisation and activation [56]. Kinetic analysis of PHD activity based on published studies shows that these enzymes greatly depend upon the concentrations of the two limiting substrates 2-OG and O₂, and of the 2-OG derived product, succinate and the substrate analogues citrate, fumarate, oxaloacetate, pyruvate and malate (Figure 3 and see below for further detail). Cellular succinate and fumarate concentrations in tumours with SDH or FH mutations can markedly change basal levels from less than 0.1 mM found in normal cells to increase up to 1-10 mM in cancer cells [57]. The intracellular concentrations of 2-OG [58] and citrate, pyruvate and malate [59, 60] determined in tumor cells are respectively, 2-2.3 mM, 1.7-2.5 mM, 2.1-8.5 mM and 2.1 mM. The inhibition constant (Ki) values of PHD for succinate, fumarate, citrate, pyruvate and malate [61-63] are respectively, 430 μM, 50 μM, 180 μM, and 1.2 mM. Succinate mediated PHD inhibition is often detected in neuroendocrine tumours with SDH mutations which show nuclear stabilisation of HIF-α’s even under normoxic conditions [64]. The Km values of 2-OG for human PHD are in the range of 55–60 μM [65]. A higher concentration of 2-OG is also known to weaken the succinate mediated inhibition of PHD and hence, the balance of these two TCA metabolites regulates PHD activity in cancer cells. Thus, in vitro studies have demonstrated the reversal of the PHD inactivation caused by succinate when the cells were provided with excess 2-OG [49], indicating that product inhibition is regulated by the ratio of 2-OG to succinate, as these compounds directly compete with each other for PHD binding and regulation [66].

The accumulation of succinate levels is a common mechanism for HIF-1α stabilisation most often detected in tumours harbouring mutations in SDH genes like pheochromocytoma and paraganglioma [67], cutaneous leiomyomas, leiomyosarcoma, and renal cell carcinoma [68, 69], but may also apply commonly to progression of other cancers as well. FH is a tumour suppressor gene whose mutation has been associated with uterine and cutaneous leiomyomas, leiomyosarcoma, and renal cell carcinoma [68, 69]. Mutation in the FH gene causes an increase
in the cytosolic concentration of fumarate, which in turn activates hypoxic signals by inhibition of the 2OG-dependent dioxygenases such as the PHD genes, resulting in HIF-1α stabilisation [67]. Despite its primary role as a mitochondrial gene, FH is also present in the cytosol, as it is dual targeted to both the cytosol and the mitochondria. Whilst the mitochondrial function of FH is to convert fumarate to malate and vice versa [70], it has a different cytosolic function as a DNA damage response protein. This additional role is not surprising given that it is also a tumour suppressor gene [70]. Mutation in the gene encoding ICD, the enzyme catalysing the formation of 2-OG by decarboxylating isocitrate affects the ratio of these metabolic intermediates in the same way as mutations in the SDH gene. Hence, decreased 2-OG with increasing succinate (and/or citrate, fumarate, pyruvate, malate) levels will trigger HIF-1α activity, thereby leading to a state of pseudohypoxia [71, 72].

Besides the association of pseudohypoxia with defective genes encoding mitochondrial enzymes, mutated VHL can also cause pseudohypoxia [73, 74]. However, pseudohypoxia in VHL defective renal clear cell carcinomas operates by a slightly different mechanism because these tumours show consistent HIF-2α overproduction and stabilisation, but not HIF-1α [75]. HIF-2α then likely interacts with the Myc pathway for cell cycle arrest and tumour progression [21]. Given the role of mitochondrial metabolites in regulating PHDs and inducing pseudohypoxia, it is still unclear which comes first and whether the mutation leads to the cancer malignancy or whether the cancer becomes established first before the onset of the mutations in TCA cycle genes. Another question is why are these phenomenon restricted to cancers of neuroendocrine origin?

**Kinetic analysis of the influence of TCA cycle intermediates on PHD activity**

We have undertaken kinetic analysis to explain the influence of the TCA cycle intermediates on PHD activity detected in vivo, by using a simulation model assuming a kinetic Bi-Bi ordered mechanism for binding of 2-OG and then O2 to the enzyme, and further release of first CO2 followed by succinate [76] (see rate equation below). Assumptions included a maximal velocity ($V_{max}$) value of 10 nmol min$^{-1}$ mg$^{-1}$, under saturating levels of Fe$^{2+}$, peptide (i.e., HIF-1α) and ascorbate. The $K_m$ values of PHD for 2-OG (A) and O2 (B) are 60 μM and 230 μM, respectively [65], with the $K_i$ values for succinate, fumarate, citrate [61] and pyruvate as respectively, 430 μM, 50 μM, 180 μM and approximately 500 μM. The PHD activity was modeled at the physiological concentrations of 2 mM 2-OG existing in cancer cells [58] with different levels of the other metabolites, using the Microcal Origin v. 5 software, as follows:

1. At low succinate concentration of 0.1 mM determined in heart [77] or
2. At higher succinate concentration of 10 mM as determined in tumors with FH mutations [57] or
3. 0.1 mM succinate and a lower fumarate concentration of 1 mM or
4. A high concentration of 10 mM fumarate which are metabolite levels found in tumors with FH mutations [57] and
5. with multiple inhibitors, using 1 mM succinate plus 1 mM fumarate plus 2 mM citrate plus 2 mM pyruvate. The results from this modeling are presented in Figure 3.

$$v = \frac{V_{max}([2OG][O2])}{K_m[2OG] \cdot K_m[O2]} \left( \frac{1}{1 + \frac{[2OG]}{K_m[2OG]} + \frac{[2OG][O2]}{K_m[2OG] \cdot K_m[O2]} + \frac{[Fum]}{K_{Fum}} + \frac{[Cit]}{K_{Cit}} + \frac{[Pyr]}{K_{Pyr}} + \frac{[CO2][Succ]}{K_m[CO2] \cdot K_{Succ}} + \frac{[Succ]}{K_{Succ}}} \right)$$
The relationship of intra-tumoral hypoxia to angiogenesis and metastasis

Recurrence and metastatic spread from residual disease after initially removing primary solid tumours and draining lymph nodes remains a major clinical problem, with many cancers recurring at high frequency [78, 79]. One of the major contributing factors to tumour metastasis is the process of neovascularization, which occurs as an outcome of the induction of hypoxia regulated proteins [80]. It is well known that intratumoural hypoxia is one of the major factors that drives tumour angiogenesis, and hypoxia-driven angiogenesis is primarily mediated by HIF-1α, often considered as a master regulator of angiogenesis in hypoxia [81]. HIF-2α also contributes to hypoxia driven angiogenesis by regulating the expression of VEGF, VEGF receptors (VEGFR) 1 and 2 and angiopoietins needed for the development of blood vessels [82]. HIF-2α triggers the chronic hypoxia response and acts as a key inducer of genes involved in tumour invasion such as the matrix metalloproteinases (MMPs) [83] and stem cell factor OCT 4 [21]. The HIF regulated genes provide dual protection to the growing tumour involving firstly, promotion of the development of new blood vessels (see Figure 1) and secondly, by helping the tumour to metabolically acclimatize to the decreased levels of O₂ [15]. Myc oncogenic activation collaborates with HIF-2α expression and causes the tumour cells to adapt the metabolic shift from oxidative phosphorylation to substrate level phosphorylation occurring within the tumour milieu under prolonged hypoxia [7, 21, 84]

One key question that arises is what is the driver that makes tumour cells migrate to form metastases? Within the heterogeneous tumour exists a metastatically predisposed subpopulation of tumor cells [85] that are exposed to the hypoxic microenvironments and as a result undergo an EMT [86]. These phenotypic changes are brought about by Myc-mediated activation of the Snail transcription factor [87]. Myc also contributes to cellular migration and invasion by altering cell-cell matrix interactions as a transactivator of the LGALS1 gene expression as well as cytoskeletal remodelling via Myc-activated RhoA expression [88]. Activation of c-Myc and the associated glycolytic shift [89], together with slower electron transfer and O₂ consumption in the mitochondrial respiratory chain, stimulates the production of reactive oxygen species (ROS) [90]. However, HIF-2α induction by Myc helps to shield the cells from the harmful effects of ROS by activating those genes responsible for the production of antioxidants [91]. HIF-1α counteracts the effects of antioxidants by triggering the calpain dependent degradation of HIF-2α [91]. The resulting decrease in HIF-2α protein levels promotes p53 serine15 phosphorylation, thereby hampering cellular redox homeostasis [92]. ROS induction, besides causing DNA damage, also inhibits apoptosis and disables the P53 protein pathway which otherwise regulates homeostasis against cellular stress [93]. Deregulated c-Myc mediated DNA damage occurs prior to the S phase of the cell cycle, enabling unrepaired DNA to remain present during the cell cycle causing genomic instability, one of the hallmarks of cancer leading to tumour invasion and dissemination from its primary site [93]. Tumour cell mobilisation is also brought about by secretion of MMPs by tumour stromal cells such as adipocytes and carcinoma associated fibroblasts (CAFs), which brings about the digestion of the extracellular matrix [94]. This dissemination of tumour cells helps to initiate the process of metastasis leading to organ specific homing of the migratory tumour cells, a process governed by chemotactic interactions [95]. In this regard, it is interesting that HIF-2α displays opposing roles in tumour development to HIF-1α and this relationship is an area of current research focus.

Relationship between hypoxia and the induction of EMT

EMT is a crucial phase in embryological development and cancer metastasis during which the epithelial cells lose their polarity switching to adopt migratory mesenchymal cell
phenotypes [96]. In order to achieve this, the cells change their cellular signalling pathways [97]. Loss of the E-cadherin protein content and overexpression of Snail are hallmarks of EMT and increased levels of Snail, in itself, are indicative of EMT, the levels of which correlate with the degree of clinical aggressiveness displayed by tumours [98]. HIF-1α induced by hypoxia has been found to promote EMT in many human malignancies in which the hypoxic microenvironment promotes overexpression of Snail, while attenuating the expression of E-cadherin, leading to EMT and increased cancer aggressiveness [99]. Cancer cell lines of epithelial origin, including HT-29 (colon carcinoma), MCF-7 (breast carcinoma), HepG2 (human hepatoblastoma) and PANC-1 (pancreatic carcinoma), when subjected to hypoxic conditions develop EMT associated characteristics such as overexpression of Snail, repression of E-cadherin and the nuclear localization of β-catenin [100], (Figure 2).

HIF-1α stabilisation activates Twist, a transcription factor that regulates early embryonic mesoderm formation and gastrulation, which also facilitates tumour development and metastasis. HIF-1α induces Twist gene expression via an HRE present in the proximal promoter region [101]. It has been proposed that the EMT pathway in tumours differs depending on whether the level of hypoxia is acute or chronic, thereby affecting the extent of protein expression and recruitment. Acute hypoxia upregulates Twist and has a reversible effect on EMT whereas more chronic, long term hypoxia results in an irreversible EMT via the activation of the ZEB2 zinc finger binding protein [102]. The NOTCH signalling protein is a mediator in the convergence of hypoxic responses to promote the EMT signal [103]. The HIF-1α-mediated EMT pathway still remains undefined as to the precise mechanisms operating to regulate this multi-gene/protein interactive cascade.

The hypoxic tumour microenvironment aids cancer cells to escape immunosurveillance

The immune responses occurring within the tumour microenvironment represent another critical aspect for the important role of hypoxia in tumour biology. Immune cells can recognise and eliminate cancer or precancerous cells based on the types of tumour associated or tumour specific antigens that these malignant cells can present. However, tumours arise more readily when the cancer cells overcome the ability of the immune system to eliminate them by processes involving immune-editing and tumour-mediated immune suppression [104]. Despite decades of research, the mechanisms for the immune suppression within the tumour microenvironment are still not completely understood. The emerging evidence suggests that intratumoral hypoxia may also be a major contributor to the immunosuppressive microenvironment. Thus, hypoxia can systemically impair the tumour antigen-specific immune responses from being produced within a tumour [105]. Firstly, the hypoxic state suppresses maturation of the dendritic cells by inhibiting CD40 and MHC class II expression, which is associated with diminished Th1 responses, including decreased Th1 cytokines, IFN-γ, TNF-1α and IL-12 expression [106].

Studies have shown that intratumoral hypoxia promotes negative immunoregulatory cell populations to exist within tumours. Thus, hypoxia-exposed tumour cells produce greater levels of the CC-chemokine ligand 28 (CCL28), which selectively attracts and recruits regulatory T (Treg) cells into the tumour stroma, negatively regulating anti-tumour immune cell responses [107]. Furthermore, CD39 (ecto-apyrase) and CD73 (ecto-5′-nucleotidase) which convert ATP and ADP into adenosine, are upregulated on the intratumoural Treg cells during hypoxia [108]. As a result, the increased intratumoural levels of soluble adenosine bind via the A2A receptor on effector T cell surfaces, promoting T cell growth inhibitory signals [109]. In addition, the intratumoural hypoxic conditions recruit monocytes, mainly derived from the bone marrow into the tumour where they can rapidly differentiate into tumour-associated macrophages (TAMs). The accumulated TAMs in the hypoxic regions of tumours express tumour-promoting cytokines
as well as inhibiting cytotoxic T lymphocyte (CTL) function [110], preventing anti-tumour immune responses.

Hypoxia also directly regulates CTL function, because hypoxia-exposed CTLs are less sensitive to the antigens presented by the tumour cells [111]. By using HIF-1α-deficient CD4+ and CD8+ T cells, it was shown that these cells had a greater capacity to proliferate and to produce IFN-γ within the intratumoral hypoxic domains [112]. An additional strongly immunosuppressive relationship has been found between expression of Galectin-1, intratumoral hypoxia and defective anti-tumour immune responses [113, 114]. Galectin-1 belongs to the family of animal lectins known to block effector T cell activation and to promote their apoptosis (reviewed in [92]) and galectin-1 is linked to HIF directly in that the galectin-1 gene is HIF-inducible, containing two HREs. It has been shown that it is possible to enhance anti-cancer effector T cell responses within tumours by inhibiting galectin-1 function using disaccharides [80]. Intratumoral hypoxia can bring about metabolic reconfiguration by increased expression of lactate dehydrogenase [115, 116], inducible nitric oxide synthase (iNOS) and indolamine-2,3-dioxygenase (IDO) [117], resulting in the accumulation of lactic acid, nitric oxide [118] and kynurenine [119] within the tumour, which in turn significantly inhibits tumour-specific CTL function [117, 120]. Thus, hypoxia-mediated immunosuppression has been found to be a novel and significant mechanism for promoting tumour evasion from immune cell attack.

The intratumoral hypoxic microenvironment shields the cancer stem cells

The relationship between tumour hypoxia, post treatment relapse and distant metastasis has become well established [121]. This is likely because intratumoral hypoxia contributes to providing a heterogeneous tumour microenvironment by promoting regions containing increased numbers of cancer stem-like cells (CSCs). Many cancers are now considered to contain small subsets of stem-like cells called tumour-initiating or cancer stem cells (CSCs), whose numbers are elevated by hypoxia. These cells acquire many phenotypic characteristics with greater capacity for self-renewal, differentiation, anti-apoptotic features, anchorage independence and are highly pluripotent and thus can migrate to distant sites to initiate new tumour formation [122-125] [126, 127]. There is increasing evidence for stem cell accumulation associated with post treatment relapse occurring within tumours. Thus, a recent study by Chen et al. [126] on glioblastomas demonstrated that CSCs not only confer chemotherapeutic resistance but can also cause post treatment relapse and the propagation of new tumours. In addition, the hypoxic microenvironment within tumours has been proposed to provide a conducive CSC niche where they can be protected and maintained by fuelling them with necessary inter- and intracellular signalling (Figure 2). Stabilised HIF-1α can lead to CSC accumulation and propagation by promoting their proliferation and greater self-renewal [123, 128]. Cycles of hypoxia and re-oxygenation were shown to enhance the proliferation of human breast CSCs that were then highly tumorigenic and metastatic when implanted into immune compromised mice [86]. Therefore, it is likely that cycling hypoxia, which normally occurs within the tumour microenvironment, is an important physiological phenomenon enabling CSCs to maintain their stem-like capacity and regeneration within the heterogeneous tumour microenvironments, promoting metastasis [129].

Embryonic stem cell (ESC) markers, such as the transcription factors OCT3/4 and SOX2 are instrumental in the maintenance and self-renewal of embryonic stem cells and primordial germ cells. The expression of OCT3/4 and SOX2 are also being increasingly recognised for their roles in cancer cell survival, self-renewal, differentiation and proliferation in solid tumours including lung, gastric, colorectal, rectal, bladder, breast, prostate and ovarian cancers [130-132].
Hypoxia, through HIF-α’s induces the ESC factors as part of the altered transcriptional program promoting ESC proliferation and in many cancer cell types, hypoxia causes expression of the induced pluripotent stem cell (iPSC) factors, OCT4, NANOG, SOX2, KLF4, c-Myc (in prostate, brain, kidney, cervix, lung, colon, liver, and breast tumours) [133], (Figure 2). Recently, a HIF/hypoxic switch has been described in a number of different cancers, which proceeds initially via a transient HIF-1α activation, followed by constitutive HIF-2α expression occurring after more prolonged periods of hypoxia and this hypoxic switch to HIF-2α enhances the tumour-initiating cell population [134-136] Human cancers share a common tumorigenic trait with an obligatory growth axis requiring HIF-2α as a major genetic convergence point in cancer, because inhibiting HIF-2α expression using short hairpin RNAs (shRNA) prevents the in vivo growth and tumorigenesis of a wide range of cancers, regardless of their mutational status or tissue of origin, including highly aggressive glioblastoma, colorectal, and non–small-cell lung carcinomas and the in vitro autonomous proliferation of several others [137].

Therapeutic resistance and anticancer therapies targeting hypoxia:

Hypoxic regions within tumours develop at a distance of 100-150 μm from the available blood vessels [138]. In addition, to having inchoate vasculature, solid tumours also have fewer lymph nodes causing build-up of interstitial fluid pressure inhibiting intratumoral transport of macromolecules which restricts intratumoral drug delivery [139]. Lactic acid produced as result of hypoxic episodes generates an acidic pH which in turn decreases the efficacy of drug uptake by tumours [140]. HIF also contributes to drug resistance by irreversible cell cycle arrest, increased repair of DNA damage and inhibition of apoptosis [8] [141]. In more general terms, tumour resistance to chemotherapy can be due to drug pharmacokinetic constraints imposed by the tumour microenvironment and/or tumour cell intrinsic resistance by expression of the multidrug resistance (MDR) P-glycoproteins (aka ABC drug transporters) [142]. One question that remains is whether there is an obligatory target of HIF-α activation that is essential for the subsequent tumour progression to metastasis, because targeting HIF-α is likely to be toxic [143]. Several drug manufacturers and pharmaceutical companies have designed drugs whose aim is to selectively target the chemo- and radio-resistant hypoxic tumour cells [144]. Most drugs have been aimed at inhibiting HIF proteins directly or via the downstream HIF regulated proteins such as CA-IX or lysyl oxidase (LOX) [145].

The Food and Drug Administration (FDA) of the USA approved the camptothecin analogue, topotecan, as one of the first HIF-1α inhibiting drugs to be tested on humans, followed by PX-478 and YC-1 [144]. Hypoxic tumours can also be targeted with pro-drugs that form cytotoxic adducts under low oxygen concentrations. Thus, tirapazamine (TPZ), a hypoxia activated pro-drug, was found to be effective in mouse models but failed further development due to reports of high non-specific toxicity in Phase III clinical trials [145]. Other hypoxia activated pro-drugs such as 3,5-dinitrobenzamide-2-mustard (PR-104) have shown anti-tumour activity, especially by targeting the hypoxic regions of tumours, as they form reactive cytotoxic hydroxylamines at low oxygen concentrations [146]. Proacta’s proprietary hypoxia-activated irreversible multi-kinase inhibitor, PR610 was recently approved by the FDA for Phase I and II clinical trials being conducted in the USA and New Zealand [147]. Given that Wilson and Hay have recently reviewed targeting hypoxia in cancer therapy and have listed all the HIF activated cytotoxic pro-drugs and their clinical status [148], these approaches will not be further discussed here.
Another interesting strategy for drug delivery targeting hypoxia is by using anaerobic facultative bacteria which possess oncolytic properties and can specifically colonise only within the hypoxic regions of solid tumours. This procedure was proposed to be particularly effective as the bacteria would fail to grow under aerobic conditions and thereby offered no threat to the well-oxygenated normal tissues. One such example is the YB1 strain of Salmonella typhimurium, engineered to grow under hypoxic conditions [149]. Recombinant bacteria were able to produce tumour necrosis factor, HIF-1α antibody or other oncolytic biomolecules and are being developed into a potential tool for drug delivery within solid tumours [150].

**Summary**

The intratumoural hypoxic microenvironment favours tumour adaptability during states of low oxygen tension. An analogy would be like the renewed phoenix in Greek mythology arising from the embers of the nest. Stabilisation of HIF-α is identified as one of the major contributors to the hypoxic microenvironment, which in turn promotes a battery of events favouring cancer cell survival and progression. It is now clear that the hypoxic microenvironment favours primary tumours by helping them to overcome multiple hurdles on the pathway to malignancy. It shields some of the tumour cells, enabling them to escape attack and predation by the immune system. The mechanisms involved in these escape processes are being elucidated, although one essential feature appears to be the decreased activity of the PHD regulatory enzymes, thereby enhancing HIF-α protein stability, triggering production of factors like VEGF and EPO to increase nourishment for the expanding cell mass via neovascularization. These tumour promoting effects of HIF-α also help to maintain an expanding/renewing population of CSCs ready to be distributed much like seeds or pollen blowing in the wind. The analogy extends to these cells waiting for the right favourable opportunity or signals before planting themselves in more fertile soil elsewhere and then proceed to proliferate through the entire development and differentiation stages of cancer as distant metastases. It’s evident from the review that tumour hypoxia play a pivotal role in the tumour development, metastasis, drug resistance and post treatment relapse and hence should be included as one of the hallmarks of cancer (REF HANAHAN AND WEINBERG HERE). Further research is aimed at understanding the complex relationships of the HIF family to cancer biology and their multi-faceted roles within the hypoxic tumour microenvironment. These studies will help to drive the development of novel drugs that are capable of more precisely targeting tumours as well as the initiating cells, regardless of their staging to destroy the roots of origin, thereby eliminating the cause of cancer as a disease.

**References:**


Figure 1: Schematic representation showing HIF-α domains and their functions in mediating hypoxic regulation and relationship to different oxic states. FIH square was made larger. See addition for HIF-1/2 α
Figure 2: Schematic representation of factors involved in HIF-\(\alpha\) stabilisation and the hypoxic response triggering CSC accumulation, leading to the development of new tumours. Cytosol instead of cytoplasm.
Figure 3. Regulation of the Prolyl Hydroxylase Activity by O2 and Physiological Competitive Inhibitors of 2-oxoglutarate.

The figure indicates that at saturating levels of fumarate (High Fum), the PHD activity can be severely decreased (6-10 times) when the O₂ concentration is 5-10 μM (the hypoxic range for solid tumors). Under this last condition, the PHD activity cannot be further decreased by elevating the concentration of either succinate or the other inhibitors, because they all compete for binding to the same enzyme site. It should be also noted that the PHD activity under low succinate is low, because the O₂ concentration range examined is well below the Km value for oxygen.