“Hypoxia and HIF pathway in cancer and the placenta”

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Contributions, declarations of interest and funding sources

PSM

I declare that I participated in the authorship of this review and that I have seen and approved the final version. I have no conflicts of interest.

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JM

I declare that I participated in the authorship of this review and that I have seen and approved the final version. I have no conflicts of interest. I am in receipt of a doctoral studentship from the Ludwig Institute for Cancer Research.

CWP

I declare that I participated in the authorship of this review and that I have seen and approved the final version. I hold equity in ReOx Ltd, a University of Oxford spin-out company with interests in the development of drugs that target the HIF pathway. Work in the laboratory has received contributions from the Wellcome Trust, Medical Research Council, British Heart Foundation, Cancer Research UK, the Oak Foundation, the Ludwig Institute for Cancer Research and the NIHR Oxford Biomedical Research Centre.

AY

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Abbreviations

CCRCC – clear cell renal cell carcinoma

FasL – Fas ligand

FIH – factor inhibiting HIF

FLT1 – fms-related tyrosine kinase 1

HIF – hypoxia-inducible factor

HLA – human leukocyte antigen

MHC – major histocompatibility complex

mTOR – mechanistic target of rapamycin

NK cell – natural killer cell

PD-1 – programmed cell death 1

PD-L1 – programmed cell death ligand 1

PHD – prolyl hydroxylase domain-containing protein

PlGF – placental growth factor

VEGF – vascular endothelial growth factor

VEGFR – vascular endothelial growth factor receptor

VHL – von Hippel-Lindau

Abstract (168 words)

In this review we note that the placenta and cancer both develop in microenvironments in which there are gradients of oxygen availability. Whilst fundamentally different in that placental development is organised and physiological whilst cancer is chaotic and pathological, there are similarities in their respective capacities to proliferate, invade adjacent tissues, generate a blood supply and avoid rejection by the immune system. We provide a brief description of the hypoxia-inducible factor (HIF) pathway and indicate the ways by which HIF activity can be regulated to achieve oxygen homeostasis. We then exemplify the potential role of the HIF pathway in contributing to those functions shared between the placenta and cancer through effects on cellular proliferation, cell death, angiogenesis, blood vessel co-option, vascular mimicry, cell adhesion molecules, secretion of matrix metalloproteinases, antigen presentation mechanisms and immunosuppressive factors. We advocate future studies to explore these similarities and differences in the hope of improving our understanding of both systems and hence treatments of placental disorders and cancer.

Key words

Oxygen, hypoxia-inducible factor, placenta, trophoblast, cancer

Highlights

- The placenta & cancer both develop in environments with graded oxygen availability

- Hypoxia-inducible factor (HIF) activity is regulated via oxygen-sensitive enzymes
- Placental development is organised & physiological whilst cancer is chaotic & pathological
- The placenta & cancer both proliferate, invade adjacent tissues, generate a blood supply & avoid immune rejection
- The HIF pathway may contribute to functions shared by the placenta & cancer

Word count

2804 words

References

60

Figures

4 (3 colour; 1 black and white)

Manuscript

Introduction

The placenta and cancer

The placenta, an organ that performs the key *physiological* role of nourishing the foetus during pregnancy, shares important similarities (and key differences) with the *pathological* state of cancer. Examples include their respective capacities to proliferate, invade adjacent tissues, generate a blood supply and avoid rejection by their host's immune system; a vital distinction is that these processes are dysregulated in malignancy. It is likely that some of the molecular pathways and environmental factors that drive these phenotypes are common to both entities and, consequently, that advances in our understanding of placental physiology could yield new insights that have direct relevance to the study of cancer pathophysiology. Furthermore, such insights may highlight therapeutic opportunities for improved treatment of both placental disease and cancer. One area of fundamental importance relates to the microenvironment in which they both develop and, in particular, the availability of oxygen. Whilst one of the placenta's main functions is to ensure a steady supply of oxygen to the developing foetus, oxygen availability within solid tumours is often inadequate, leading to patchy areas of both acute and chronic hypoxia. Therefore, the process of oxygen homeostasis is a subject that merits further consideration.

Hypoxia and oxygen sensing

It has long been recognised that oxygen homeostasis is essential for the maintenance of life and that perturbations of this process play a significant role in the pathogenesis of a wide variety of diseases including tissue ischaemia/infarction, chronic lung disease, inflammatory conditions, pre-eclampsia and cancer [1-3]. Incremental research, conducted over the past three decades, has defined a molecular mechanism for sensing cellular oxygen levels that is common to all metazoan species and three key individuals have recently been awarded the 2016 Albert Lasker Basic Medical Research Award for their contributions to the discovery of this system [4]. In response to low levels of oxygen, cells up-regulate hypoxia-inducible factor (HIF), a highly-conserved transcription factor that co-ordinates an appropriate adaptive response within the physiological and pathophysiological range. HIF functions as a heterodimer of a regulatory α subunit (HIF-1α, HIF-2α or HIF-3α) and a constitutive β subunit (HIF-β) [5-8]. Hypoxia increases cellular levels of the α subunit by inhibiting the prolyl hydroxylase domain-containing proteins (PHD1, 2 and 3), oxygen, iron and 2-oxoglutarate-dependent enzymes that regulate degradation of this isoform via von Hippel-Lindau (VHL) ubiquitin E3 ligase and the proteasome [9-12] (**Figure 1**). Full transcriptional activity requires heterodimerisation and co-activator recruitment, the latter process itself regulated in an oxygen-dependent manner by the asparaginyl hydroxylase, factor inhibiting HIF (FIH) [13, 14], a member of the same extensive enzyme family as the PHDs [15] (**Figure 2**). Whilst these oxygen-sensitive processes are key, HIF activation can occur as a result of other processes that influence enzyme activity, including changes in cellular metabolism that alter iron, 2-oxoglutarate, succinate, fumarate and hydroxyglutarate levels as well as processes that affect mechanistic target of rapamycin (mTOR) and thereby influence the translational rate of HIF-α chains [12, 16-18] (**Figure 3**). Although subject to a number of negative feedback loops [19], overall activation of the different HIF isoforms leads to increased transcription of genes involved in a myriad of processes including metabolism,

angiogenesis, red cell production, neutrophil function and immunomodulatory effects (for a more comprehensive description of the HIF system we suggest reading the review by Schofield and Ratcliffe [20]). In this brief paper, we aim to summarise the relevance of oxygen and the HIF system to placental function and cancer biology (**Figure 4**).

Placental and tumour development

Fertilisation of a human ovum by a sperm results in the production of a *zygote* (a diploid cell containing both maternal and paternal chromosomes). Four subsequent cycles of cell division result in formation of the *morula*, a cluster of sixteen cells. After seven cycles of division, the embryo contains 128 cells and is now called the *blastula* (which comprises a spherical outer layer of cells [*blastoderm*] which surrounds a yolk-filled central cavity [*blastocoele*]). By the next stage of embryogenesis (*blastocyst*), an inner cell mass (*embryoblast*) has formed (which ultimately develops into the foetus) and the outer layer is composed of cells which are referred to as *trophoblasts* (which ultimately form the placenta). At this stage, 5-7 days after conception, the blastocyst attaches to the endometrial lining of the uterus and placentation begins. The gravid uterus is known to be a hypoxic microenvironment and, consequently, the HIF system plays a key role in orchestrating foetal and placental development [21]. Accordingly, mouse models in which the HIF system has been disrupted (by either *Hif-1*α and *Hif-2*α or *Hif-*β gene knockout) demonstrate embryonic lethality contributed to by abnormal placentation including inadequate invasion of the endometrium and aberrant trophoblast development [22, 23]. Interestingly, *Vhl* and *Phd2* knockout mouse models (in which HIF is stabilised due to lack of degradation), are also embryonic lethal and display a similar phenotype to their *Hif* knockout counterparts [24, 25]. Taken together, these results demonstrate that accurate control of HIF levels to produce ‘just the right amount’ is essential for normal placental development.

In a similar manner, tumours (including choriocarcinoma of the placenta) also arise from a single cell that subsequently undergoes multiple cycles of cell division. However, their development differs from that of the normal placenta in that genetic anomalies endow this founder cell with a survival advantage over its non-neoplastic neighbours. As tumour growth progresses and multiple new somatic mutations are acquired, selection pressure results in the development of related, but distinct, sub-clones within the same tumour in a Darwinian fashion [26]. During this process of clonal evolution, positive selection occurs for mutations that disrupt key physiological pathways in such a way as to drive the malignant phenotype ('driver' mutations). The tumour-promoting potential caused by dysregulation of the HIF system is best exemplified by the rare genetic condition VHL disease. In this autosomal dominant condition, affected individuals inherit a mutation in the *VHL* tumour suppressor gene on chromosome 3p. Subsequent inactivation of the wild type allele leads to the development of a variety of neoplasms, occurring in specific tissues including cerebellar and retinal haemangioblastomas, clear cell renal cell carcinomas (CCRCC) and phaeochromocytomas. Furthermore, *VHL* inactivation is also an early genetic event in the development of the majority of sporadic clear cell renal cell carcinomas. Intriguingly, HIF-1 and HIF-2 have different effects in the context of CCRCC [27, 28] and in fumarate hydratase deficiency, another genetic cancer syndrome in which the HIF pathway is dysregulated, abnormal cellular growth is not dependent on this dysregulation, at least in mouse models [29].

Placental and tumour growth

After their initiation, both the placenta and a tumour continue to grow and adjustments in a number of key cellular processes favour their expansion.

Increased cellular proliferation

Both trophoblasts and malignant cells demonstrate high rates of proliferation and a number of factors help to contribute to their rapid rate of cell division. Examples included self-sufficiency of growth factors, activation of intracellular signal transduction pathways and altered expression of cell cycle control proteins and nuclear transcription factors. Of these, several have relevance to oxygen sensing. In addition to inducing angiogenesis, vascular endothelial growth factor (VEGF) has also been shown to promote both extravillous trophoblast [30] and malignant cell [31, 32] proliferation. Both *VEGF* [33] and its receptor *VEGFR1* (also known as fms-related tyrosine kinase 1 [*FLT1*]) [34] are HIF target genes meaning, that under certain conditions, hypoxic cells have the potential to increase their rate of cell division. Furthermore, VEGFR1 can be bound and activated by placental growth factor (PlGF) [35], which is also up-regulated in cancer and chronic inflammatory diseases [36], further augmenting this autocrine and paracrine signalling mechanism.

Reduced cell death

Coupled with an increased rate of cell division, a reduction in rate of cell death also contributes to placental and tumour enlargement. Both trophoblasts and malignant cells are known to be inherently resistant to apoptosis and one mechanism that contributes to this phenomenon is expression of survivin [37, 38], an anti-apoptotic protein that blocks caspase activation. Survivin has been shown to be a HIF-1α target gene in cervical cancer cells [39], demonstrating another mechanism by which hypoxia increases tumour growth. Paradoxically, under some circumstances, hypoxia can promote *increased* trophoblast apoptosis, thus contributing to the development of a range of placental disorders including intrauterine growth restriction and pre-eclampsia [40]. Similarly, in cancer cells, hypoxia can also increase expression of BNIP3, a pro-apoptotic factor [41].

Increased blood supply

To sustain continued growth, both placentas and tumours must establish vascular networks to provide an adequate supply of oxygen and nutrients. Whilst this process is *physiological* in the placenta but *pathological* in a tumour, several shared mechanisms contribute to the development of such networks:

- Angiogenesis – as both *VEGF* and *VEGFR1* (*FLT1*) are HIF target genes, hypoxic trophoblasts and tumour cells, as well as adjacent stromal cells sharing the same microenvironment, have the capacity to initiate angiogenesis (the development of new blood vessels from pre-existing ones) in an attempt to improve local oxygenation.

- Blood vessel co-option – in a manner similar to the placenta's use of the uterine spiral arteries as its main blood supply, tumours are able to derive a blood supply from pre-existing blood vessels by a process termed 'blood vessel co-option'.
- Vasculogenic mimicry (the development of vascular channels that are lined by cells other than endothelium) – the galactose-binding protein galectin-3 is known to contribute to the acquisition of a vascular phenotype by malignant melanoma cells [42] and is also known to contribute to trophoblast differentiation [43]. Through the process of vasculogenic mimicry, it is believed that both cell types are able to directly contribute to their own blood supply.

These phenomena have important clinical implications. Firstly, the ability of tumour cells to co-opt local blood vessels and to differentiate into vascular channels themselves may contribute to resistance to anti-angiogenic therapy (reviewed in [44]). Secondly, similarities exist between the side effect profile of such treatments and pre-eclampsia (a triad of hypertension, proteinuria and peripheral oedema developing after the twentieth week of pregnancy that is associated with high maternal and foetal morbidity and mortality), implicating insufficient angiogenesis in the pathophysiology of this condition. Indeed, trophoblasts are known to produce a soluble version of Flt-1 (sFlt-1), which antagonises the effects of VEGF and is therefore a therapeutic target for the treatment of pre-eclampsia (reviewed in [45]).

Invasion of local tissues

205 At the time of implantation, trophoblasts differentiate into villous and extravillous subtypes. The villous trophoblasts fuse to form terminally-
206 differentiated, multinucleated syncitiotrophoblasts that cover the chorionic villi and participate in gas and nutrient exchange with maternal blood
207 flowing through the intervillous space. Meanwhile, the extravillous trophoblasts proliferate, leave their basement membrane and invade the
208 adjacent decidualised endometrium and superficial myometrium (*interstitial invasion*; to anchor the placenta to the uterine wall) and uterine
209 spiral arteries (*endovascular invasion*; to remodel the maternal vessels to ensure adequate blood supply to the placenta). Under normal
210 conditions, placental invasion of the endometrium is tightly regulated and dysregulated trophoblastic invasion has been implicated in the
211 pathogenesis of pre-eclampsia. Local invasion of adjacent tissues is also an early feature of tumour spread and both processes share broadly
212 similar processes mechanisms including:

213 • Changes in expression of cell surface adhesion molecules – both invasive trophoblasts and malignant cells demonstrate altered expression
214 of cell-cell (e.g. cadherins and members of the immunoglobulin superfamily) and cell-extracellular matrix (e.g. integrins) adhesion molecules
215 in ways that favour the transition from adhesive to migratory phenotypes.
216 • Production of proteinases – both cell types secrete proteinases (e.g. matrix metalloproteinases) that facilitate basement membrane
217 degradation and their migration through the surrounding extracellular matrix.

218

219 *In vitro* analyses of HIF-deficient trophoblast stem cells have elucidated some of the factors that contribute to the deficient invasion seen in the
220 corresponding knockout mouse models [46]. These cells display reduced adhesion to and migration towards the extracellular matrix protein
221 vitronectin when compared to their wild-type counterparts and this difference was associated with a reduction in surface expression of integrin

222 αvβ3 (which is known to be expressed by invasive extravillous trophoblasts and to facilitate their migration *in vivo* [47]). Interestingly, and of
223 relevance to tumour invasion, increased adhesion, migration and integrin αvβ3 surface expression were seen when the experiment was
224 repeated with B16F0 mouse melanoma cells that had been cultured in hypoxia (1.5% O_2) for 24 hours. However, unlike malignant spread,
225 trophoblastic invasion is subject to tight spatial (inner third of the uterine myometrium) and temporal (invasion is maximal during the twelfth
226 week of gestation before declining to stop by the mid-point of gestation [48]) regulation.

227

228 *Immune privilege*

229 Another feature common to trophoblasts and cancer cells is the ability to avoid destruction by the host immune system. The developing
230 placenta is a semi-allograft-like structure, containing paternally derived antigens capable of generating an adaptive response from the maternal
231 immune system. Likewise, cancer cells express a range of neoantigens, including mutated peptide epitopes, derived from their inherent genetic
232 instability and in some cases viral proteins. Immune escape is now recognised as one of the key hallmarks of cancer development [49] and a
233 growing body of evidence is implicating oxygen-sensing pathways in this process (reviewed in [50]). Mechanisms of immune evasion fall into
234 two broad strategies: invisibility (avoiding recognition by the immune system) and blockade (up-regulating mechanisms to limit the action of
235 immune cells). The specifics of immune tolerance in each site have been extensively reviewed elsewhere (cancer reviewed in [51], cancer and
236 placenta reviewed in [52]) so we will focus on some key examples and their relation to the HIF pathway.

237

Invisibility is broadly achieved through loss of antigen-presentation mechanisms. It is estimated that 40-90% of human tumours derived from major histocompatibility class I (MHC-I) positive tissues have clonally selected for loss of MHC-I expression, removing tumour-antigens from the cell surface and thus protecting against T cell-mediated immunity [53]. Similarly, trophoblasts lack expression of the highly polymorphic human leukocyte antigens (HLA-A, B and C) and instead express non-classic HLA-G, which has the effect of protecting against natural killer (NK) cell attack, a phenomenon also observed in some cancer cells. HLA-G is hypoxia-inducible and directly regulated by HIF-1α [54].

Immune blockade is achieved by the up-regulation of immunosuppressive factors to directly perturb local immune cell function. There is huge diversity in site-specific immune regulatory pathways, with many likely yet to be elucidated. Common to both the placenta and malignant tumours is increased expression of Fas ligand (FasL), the ligand for the cell surface death receptor Fas. FasL is up-regulated by trophoblasts at the placental/maternal interface and is also up-regulated in solid tumours, particularly following chemotherapy. FasL expression functions to generate immunologically privileged sites, inducing the apoptosis of Fas expressing infiltrating lymphocytes. Mouse models of alveolar injury have indicated a role for HIF-1α in regulating FasL/Fas mediated apoptosis [55]. Acting in a similar manner, expression of PD-L1, a member of the B7 checkpoint gene family and a potent negative regulator of T cell responses, is increased on cells in both the placenta and tumours, thereby contributing to immune tolerance in both settings [56]. Engagement of programmed cell death ligand 1 (PD-L1) with its receptor (programmed cell death 1 [PD-1]) induces apoptosis of T cells or renders them refractory to activation. Recent research has demonstrated HIF-dependent regulation of PD-L1 expression in cancer [57]. Similarly, temporal expression of PD-L1 on trophoblasts throughout placental

development has been linked to differential oxygen availability [58]. Mechanisms such as those outlined above indicate that oxygen-sensing pathways play a key role in immune regulation and this area requires further investigation.

Conclusions

Despite the high complexity of cellular physiology and differences in gene expression between distinct cell types, it is perhaps unsurprising that the broadly similar phenotypes of extravillous trophoblasts and cancer cells are driven by overlapping, 'hardwired' physiological pathways such as the HIF system. A critical difference is that placental development is a highly regulated process whereas a state of dysregulation characterises all of the key hallmarks of carcinogenesis [49]. Nevertheless, trophoblasts also have the potential to undergo malignant transformation. Choriocarcinoma is a malignancy of the placenta that most commonly develops on a background of hydatidiform mole (an abnormal pregnancy derived from a fertilised but non-viable ovum with gross chromosomal abnormalities). Consistent with the behaviour of normal trophoblasts, choriocarcinoma cells invade the uterine wall aggressively, frequently demonstrate lymphovascular spread and often produce distant metastases. Interestingly, *in vitro* studies have shown that HIF accumulation within choriocarcinoma cells promotes metabolic [59] and migratory [60] changes that favour tumour growth and spread.

Future studies of extravillous trophoblast physiology should place a key emphasis on the mechanisms that control the replicative and invasive capacities of these cells, since such work might identify molecular targets with relevance for the treatment of a broad range of solid tumours. At

the same time, a better understanding of hypoxia sensing in both benign and neoplastic trophoblasts will facilitate a deeper understanding of the pathophysiology of placental disorders, including pre-eclampsia and choriocarcinoma, and hopefully improve treatment of these rare but devastating conditions.

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Figure legends

Figure 1 – action of the prolyl hydroxylase domain-containing proteins (PHD1-3)

Hypoxia increases cellular levels of HIF-α subunits by inhibiting PHD1-3, oxygen, iron and 2-oxoglutarate-dependent prolyl hydroxylases. Under normoxic conditions, these enzymes hydroxylate two critical prolyl residues (P402 and P564 in human HIF-1α), allowing the VHL ubiquitin ligase to target the subunit for proteasomal degradation. Inhibition of this process under hypoxic conditions (or alterations in iron or 2-oxoglutarate availability) leads to HIF-α stabilisation.

(*bHLH – basic helix-loop-helix motif, CO_2 – carbon dioxide, Fe^{2+} – ferrous, HIF – hypoxia-inducible factor, O_2 – oxygen, PAS – Per-ARNT-Sim domain, PHD – prolyl hydroxylase domain-containing protein, TAD-C – C-terminal transactivation domain, TAD-N – N-terminal transactivation domain, VHL – von Hippel-Lindau protein*)

Figure 2 – action of factor inhibiting HIF (FIH)

Full transcriptional activity requires heterodimerisation and co-activator recruitment, the latter process itself regulated in an oxygen-dependent manner by the asparaginyl hydroxylase FIH. Under normoxic condition, this enzyme hydroxylates an asparaginyl residue (N803 in human HIF-1α), preventing recruitment of the CBP/p300 co-activators. Inhibition of this process under hypoxic conditions (or alterations in iron or 2-oxoglutarate availability) facilitates co-activator recruitment.

(*bHLH – basic helix-loop-helix motif, CBP – CREB-binding protein, CO_2 – carbon dioxide, Fe^{2+} – ferrous, FIH – factor inhibiting HIF, HIF – hypoxia-inducible factor, O_2 – oxygen, PAS – Per-ARNT-Sim domain, TAD-C – C-terminal transactivation domain, TAD-N – N-terminal transactivation domain*)

Figure 3 – factors influencing hypoxia-inducible factor (HIF) system activity

In addition to hypoxia, many other factors affect HIF activity including processes that alter HIF-α chain synthesis and degradation as well as those that influence enzyme function.

(*HIF – hypoxia-inducible factor, mTOR – mechanistic target of rapamycin*)

Figure 4 – Hypoxia and hypoxia-inducible factor (HIF) system in cancer and the placenta

Stabilisation of HIF-α subunits (for example, in hypoxic conditions) leads to heterodimerisation with β subunits and recruitment of co-activators (CBP/p300) to form a transcription complex that binds to hypoxia response elements in the promoter regions of target genes (containing an RCGTG base sequence) and hence activation of a myriad of homeostatic processes. Exemplar references for these phenomena in cancer and placenta are listed.

(*CBP – CREB-binding protein, HIF – hypoxia-inducible factor*)

Figure 1

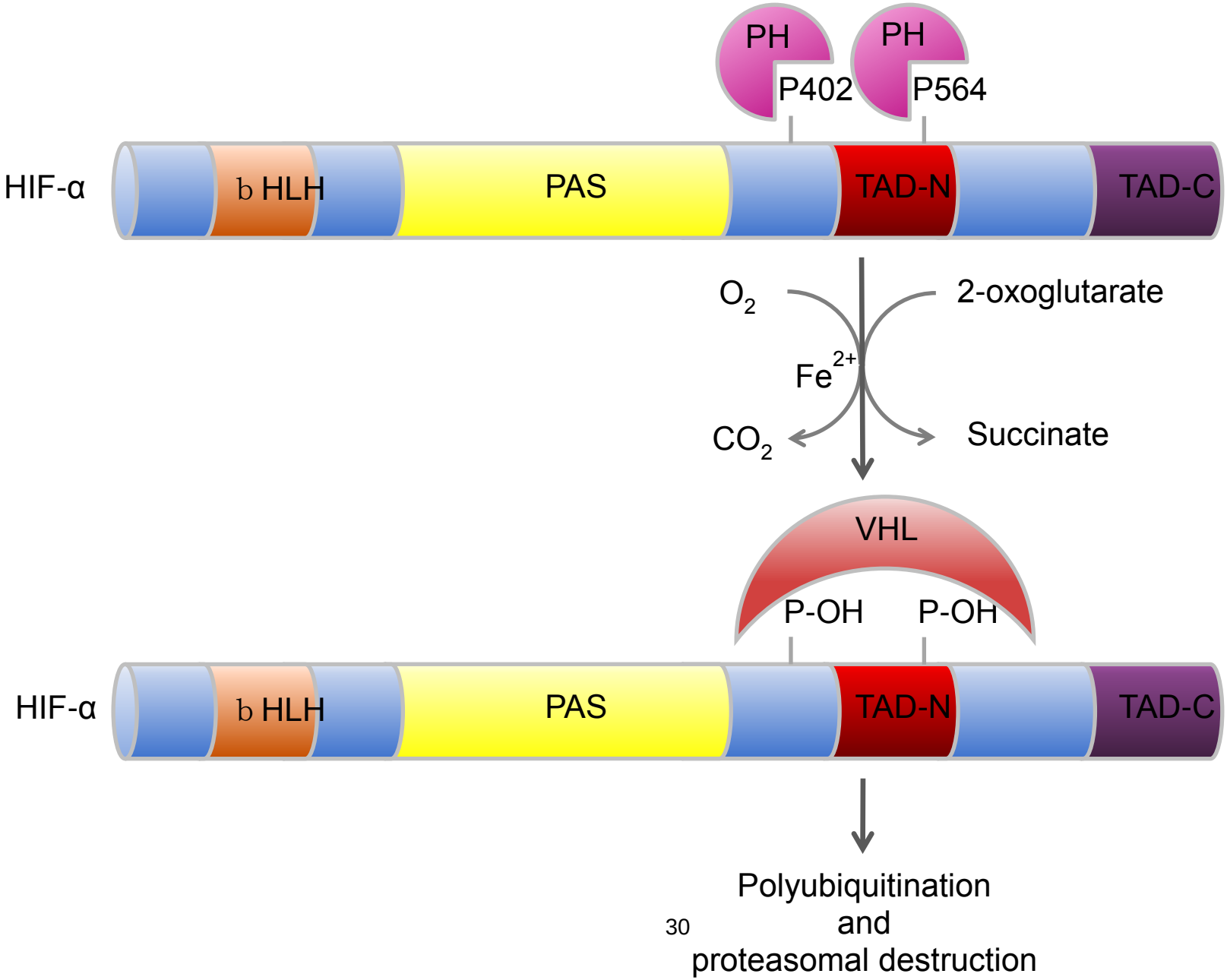

PH
PH
P402
P564
HIF-α
b HLH
PAS
TAD-N
TAD-C
O2
2-oxoglutarate
Fe2+
CO2
Succinate
VHL
P-OH
P-OH
HIF-α
b HLH
PAS
TAD-N
TAD-C
Polyubiquitination
and
proteasomal destruction

Figure 2

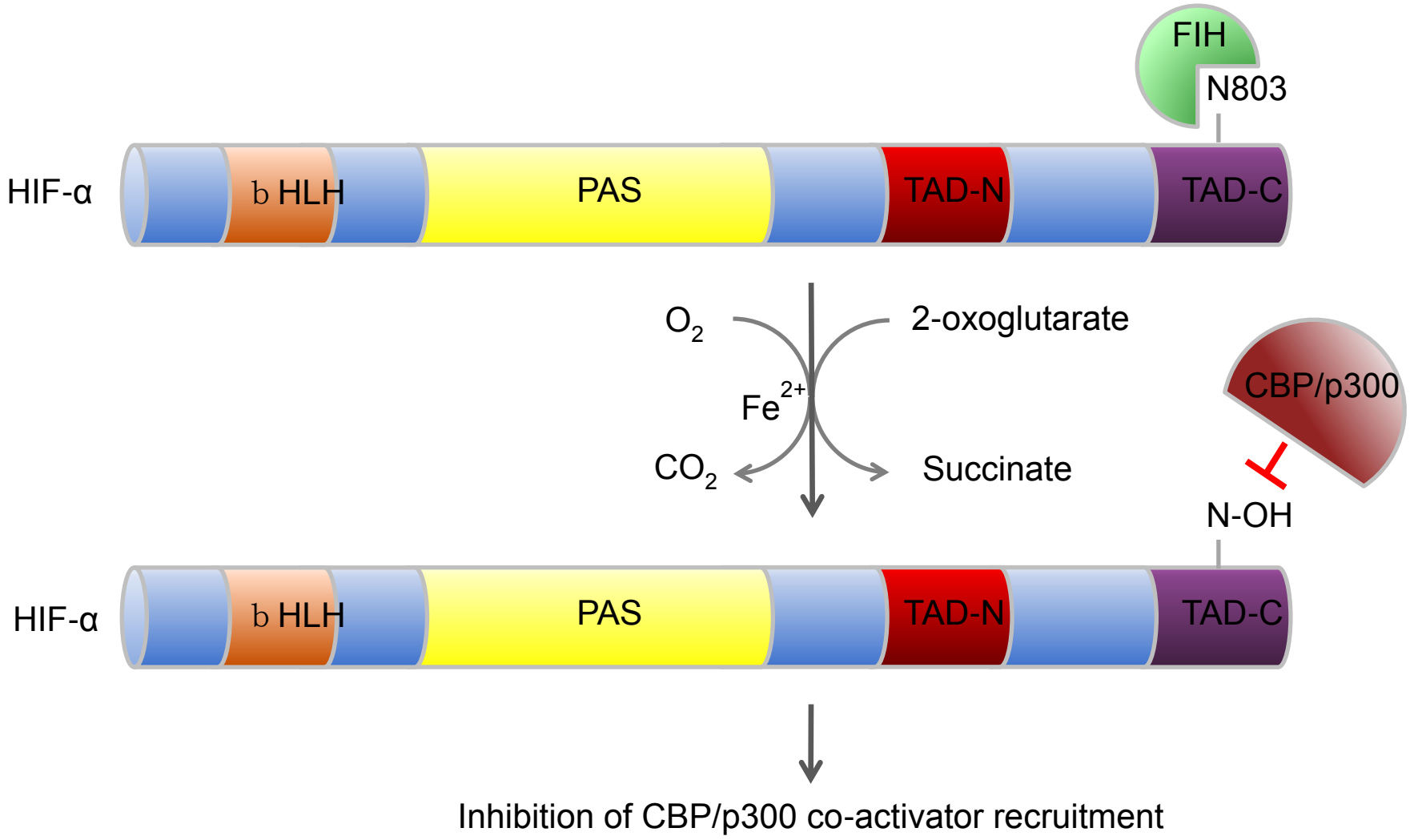
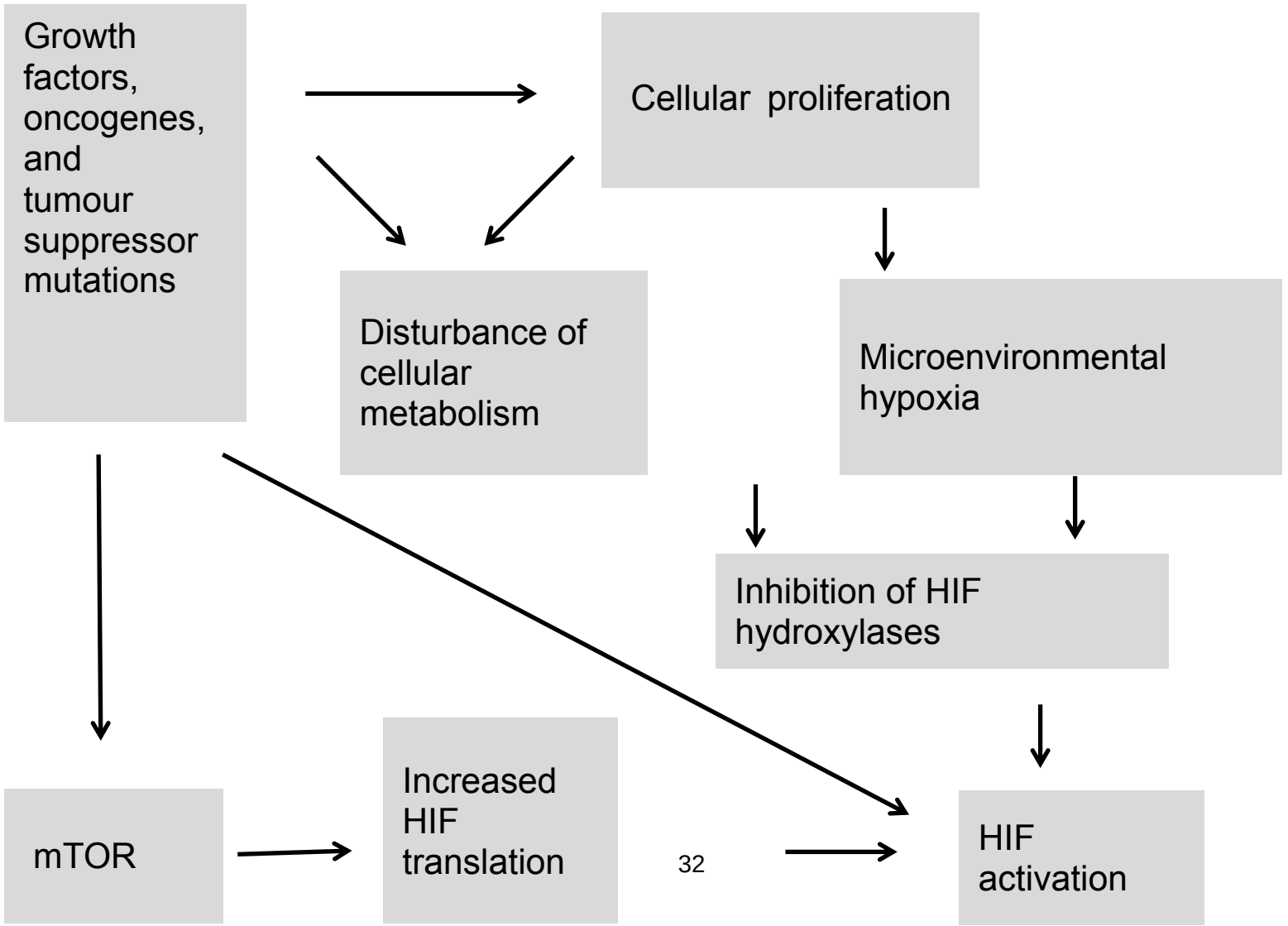

FIH
N803
HIF-α
b HLH
PAS
TAD-N
TAD-C
O2
2-oxoglutarate
Fe2+
CO2
Succinate
CBP/p300
N-OH
HIF-α
b HLH
PAS
TAD-N
TAD-C
Inhibition of CBP/p300 co-activator recruitment

Figure 3


Growth factors, oncogenes, and tumour suppressor mutations
Cellular proliferation
Disturbance of cellular metabolism
Microenvironmental hypoxia
Inhibition of HIF hydroxylases
mTOR
Increased HIF translation
HIF activation

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483 Figure 4

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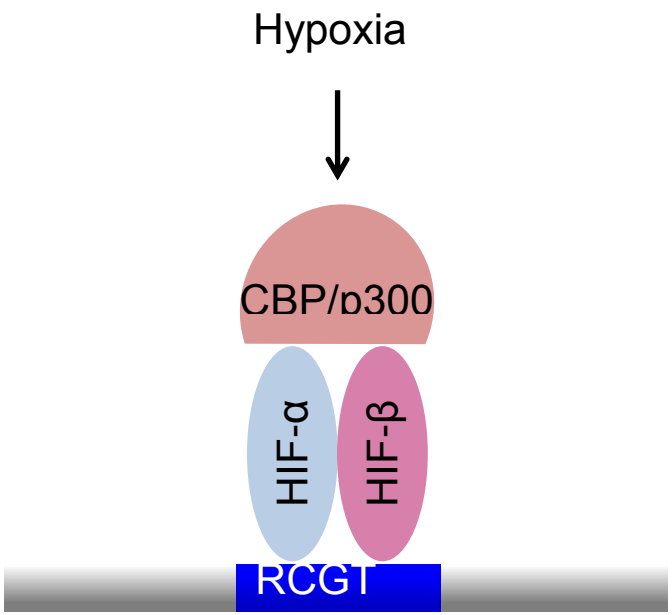

Hypoxia-inducible factor-1a

Hypoxia-inducible factor-2a

Increased transcription leading to:	*Cancer*	*Placenta*
• Increased cellular proliferation	[31, 32]	[30]
• Reduced cell death	[37, 39]	[38]
• Vascular remodelling	[45]	[45]
• Invasion of local tissues	[46]	[46, 47]
• Immune privilege	[57]	[58]