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Beyond oxygen: complex regulation and activity of hypoxia inducible factors in pregnancy

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In the first trimester the extravillous cytotrophoblast cells occlude the uterine spiral arterioles creating a low oxygen environment early in pregnancy, which is essential for pregnancy success. Paradoxically, shallow trophoblast invasion and defective vascular remodelling of the uterine spiral arteries in the first trimester may result in impaired placental perfusion and chronic placental ischemia and hypoxia later in gestation leading to adverse pregnancy outcomes. The hypoxia inducible factors (HIFs) are key mediators of the response to low oxygen. We aimed to elucidate mechanisms of regulation of HIFs and the role these may play in the control of placental differentiation, growth and function in both normal and pathological pregnancies. The Pubmed database was consulted for identification of the most relevant published articles. Search terms used were oxygen, placenta, trophoblast, pregnancy, HIF and hypoxia. The HIFs are able to function throughout all aspects of normal and abnormal placental differentiation, growth and function; during the first trimester (physiologically low oxygen), during mid-late gestation (where there is adequate supply of blood and oxygen to the placenta) and in pathological pregnancies complicated by placental hypoxia/ischemia. During normal pregnancy HIFs may respond to complex alterations in oxygen, hormones, cytokines and

growth factors to regulate placental invasion, differentiation, transport and vascularization. In the ever-changing environment created during pregnancy, the HIFs appear to act as key mediators of placental development and function and thereby are likely to be important contributors to both normal and adverse pregnancy outcomes.

Key words: oxygen / hypoxia / placenta / hypoxia inducible factor / trophoblast

Introduction

Placentation requires the complex regulation of trophoblast proliferation, differentiation and invasion. This enables the trophoblasts to breach the uterine luminal epithelium and colonize the endometrium and its vasculature in order to sequester a blood supply for the developing placenta. Impaired placental invasion, development and function have been implicated in several complications of pregnancy, such as unexplained miscarriage (Khong *et al.*, 1987), pre-eclampsia, intrauterine growth restriction (IUGR) (Khong *et al.*, 1986), placental abruption (Dommisse and Tiltman, 1992), pre-term labour with intact membranes (Kim *et al.*, 2003), premature rupture of the membranes (Kim *et al.*, 2002) and stillbirth (Smith *et al.*, 2004).

In humans, endovascular cytotrophoblasts (CTBs) invade and initially plug the uterine spiral arterioles, so there is little to no maternal blood flow to the intervillous space (IVS). Paradoxically, despite oxygen being essential for late gestation placental function and fetal growth, the early placenta and embryo differentiate in a relatively low oxygen environment. However, at about 11 weeks of gestation the arteriole plugs are displaced and maternal blood flow commences (Burton *et al.*, 1999). Extravillous CTBs (EVTs) replace the maternal endothelium and the spiral arterioles are remodelled, dramatically increasing in diameter and compliance and are no longer responsive to vasoactive molecules in the maternal circulation. This and expansion of the uterine vascular tree, permit the 12-fold increase in uterine blood flow that occurs from the non-pregnant state to provide for the demands of the growing fetus later in gestation (Martin, 1965). However, if trophoblast differentiation, invasion and/or vascular remodelling are impaired, pregnancy complications can occur. In pre-eclampsia and some miscarriages, for example, insufficient trophoblast invasion and vascular remodelling in the first trimester paradoxically promote premature blood flow into the IVS leading to reduced maternal blood flow to the placenta later in pregnancy (Khong *et al.*, 1987; Rinkenberger *et al.*, 1997; Zhou *et al.*, 1998; Jauniaux *et al.*, 2003a, b). Hypoxia is therefore implicated as a key regulator of placental morphogenesis and function. In normal pregnancy, a low oxygen environment in the placenta is physiological and necessary in the first trimester but when it occurs later in gestation it is pathological and associated with common complications of pregnancy.

Hypoxia inducible factors (HIFs) are key regulators of placental vascularization and invasion as well as trophoblast differentiation and thus are essential to placental development. It is well known that HIFs mediate the response to hypoxia, however, it is increasingly apparent that this regulation is highly complex and, furthermore, that these transcription factors can be regulated by non-hypoxic stimuli. Therefore, in addition to being potential mediators of the effects of variation in oxygen supply on placentation, HIFs may also be involved in the regulation of placental development through their interactions with hormones, growth factors and cytokines. This review will examine what is known about the regulation of placental development and function by oxygen in both normal and abnormal pregnancies. Furthermore, it will elucidate mechanisms of acute and prolonged hypoxic regulation, as well as non-hypoxic regulation, of HIFs and what role these may play in the control of placental differentiation, growth and function in both normal and pathological pregnancies. In addition, we will identify novel interactions which may regulate placental development and suggest new areas for research.

Methods

Hypoxic regulation of HIF activity in other systems has been extensively reviewed over many years. Therefore, we provide a brief overview of this area. An extensive online search of published articles on HIFs and oxygen in the placenta was undertaken. The Pubmed database was consulted using combinations of the following search terms: placenta, trophoblast, pregnancy, HIF, oxygen, hypoxia, non-hypoxic regulation, angiogenesis, invasion and transport. Where possible we have limited publications to those involving human placentation. The articles were then selected based on relevance and quality after discussion between the authors. References of selected articles were also hand searched for additional relevant citations. HIF is thought to be regulated at the post-translational level and thus articles reporting HIF nuclear localization or DNA binding were given greater weighting. However, as reported in this review, emerging evidence suggests that HIF may also be regulated at the transcriptional level and we felt that these reports, although scarce, were highly novel and worth inclusion, despite the lack of data on HIF nuclear localization or DNA binding.

Hypoxia inducible factors

HIFs are a family of basic Helix–Loop–Helix transcription factors that mediate the response to changes in cellular oxygen. HIF-1 is a heterodimer consisting of a HIF-1α subunit bound to the aryl hydrocarbon nuclear translocator (ARNT), which is also known as HIF-1β (Wang and Semenza, 1995; Safran and Kaelin, 2003). Following hypoxia-induced nuclear translocation and dimerization, HIF is able to bind to the hypoxia response element (HRE) of target genes, which has the core consensus sequence 5′-CGTG-3′, in responsive genes (Rajakumar and Conrad, 2000). HIFs mediate the expression of a wide variety of genes that play critical roles in erythropoiesis, angiogenesis, glucose transport and glycolysis (Wenger and Gassmann, 1999; Safran and Kaelin, 2003; Rocha, 2007). In mammals, three genes have been shown to encode HIF-α subunits. The expression of HIF-1α is ubiquitous whereas the expression of its paralogs, HIF-2α (Ema *et al.*, 1997; Flamme *et al.*, 1997; Tian *et al.*, 1997) and HIF-3α (Gu *et al.*, 1998) are more restricted (Wenger and Gassmann, 1999; Semenza, 2000; Daikoku *et al.*, 2003). These data indicate that HIF-1 plays a general role in homeostasis whereas HIF-2 and HIF-3 may play more specialized roles (Wenger and Gassmann, 1999; Semenza, 2000).

Oxygen-regulated HIF stabilization

The process of HIF-1α activation has been studied extensively over the past few years and includes both enhanced protein stability and transcriptional activity. In well-oxygenated cells, HIF-1α has a half-life of <5 min (Wang *et al*., 1995). Under normoxic conditions the α subunit is hydroxylated at conserved proline residues (HIF-1α Pro^{564} and Pro^{402}) (Ivan *et al*., 2001; Jaakkola *et al*., 2001; Masson *et al*., 2001) contained within a unique oxygen-dependent degradation domain that critically controls protein stability (Huang *et al*., 1998; Srinivas *et al*., 1999; Huang and Bunn, 2003; Chan *et al*., 2005). The enzymes promoting the hydroxylation of these residues are prolyl-4-hydroxylases (PHDs) of which there are three isoforms, PHD1, PHD2 and PHD3 (Bruick and McKnight, 2001; Epstein *et al*., 2001; Jaakkola *et al*., 2001). These enzymes have an absolute requirement for Fe(II) as a cofactor, and molecular oxygen as a co-substrate, but do not require ATP or NAD (P) (Jaakkola *et al*., 2001). The requirement of the PHD for molecular oxygen means that this reaction can act as an 'oxygen sensor' (Mole *et al*., 2002).

Once the proline residues of HIF-1α are hydroxylated, the von Hippel–Lindau (VHL) tumour suppressor gene product (pVHL) is able to bind (Ivan *et al*., 2001; Jaakkola *et al*., 2001; Mole *et al*., 2002). pVHL is then able to regulate HIF-1α proteolysis (Maxwell *et al*., 1999) by acting as the recognition component of an E3 ubiquitin protein ligase complex (Lisztwan *et al*., 1999; Cockman *et al*., 2000; Ohh *et al*., 2000) to mediate polyubiquitination and proteasomal degradation of HIF-1α under normoxic conditions (Salceda and Caro, 1997; Huang *et al*., 1998; Kallio *et al*., 1999; Tanimoto *et al*., 2000).

Regulation of HIF activity

The second means of HIF inhibition during normoxia involves the hydroxylation of a conserved asparagine residue (Lando *et al*., 2002a, b) by factor inhibiting HIF-1 (FIH-1), an asparaginyl hydroxylase (Mahon *et al*., 2001; Hewitson *et al*., 2002; Lando *et al*., 2002a, b). Therefore, the hypoxic regulation of HIF requires firstly the stabilization of the HIF-1α subunit and secondly its activation (Minet *et al*., 2000). Like other known hydroxylase enzymes, FIH-1 is an Fe(II)-dependent enzyme that uses molecular oxygen to modify its substrate (Lando *et al*., 2002a, b). When oxygen is available, FIH-1 hydroxylates the α subunit, inhibiting the interaction of HIF-1α and HIF-2α with the transcriptional co-activators p300/CBP whereas during hypoxia the non-hydroxylated HIF is free to bind to p300/CBP (Arany *et al*., 1996; Dames *et al*., 2002; Freedman *et al*., 2002; Lando *et al*., 2002a, b).

In addition to FIH-1, other co-factors can impact on HIF function. Specifically, p300/CBP interacting transactivator with ED-rich tail 2 (CITED2) is a negative regulator of HIF function. CITED2 actively competes with HIF-1 for p300/CBP binding (Freedman *et al*., 2003). Furthermore, *Cited2* expression is activated through a HRE in its promoter under hypoxic conditions (Bhattacharya *et al*., 1999) which suggests a possible negative feedback to HIF transcriptional activity. CITED2 may play an important role in regulating HIF function during placentation as CITED2 null mice have impaired trophoblast differentiation and fetal vascularization of the placenta (Withington *et al*., 2006).

Placental oxygenation

There has been much debate in recent years as to whether the term hypoxia should be used when discussing the oxygen tension in the placenta. Therefore, it is important to outline how we perceive hypoxia and normoxia during placental development. For the purposes of this review we have used the term hypoxia only when the oxygen concentration is lower than that to which the placenta is normally exposed. For example, as will be discussed, the first trimester placenta normally develops in a low oxygen environment and so in this case the oxygen tension is physiologically normoxic, even though it is lower than that experienced later in gestation. This is in contrast to, for example, a term placenta that is experiencing abnormally low oxygen levels and is therefore classified as hypoxic or ischemic.

Physiologically low oxygen in early placental development

In 1987, Hustin and Schaaps reported that maternal blood flow to the IVS was not fully established until ~ 11–12 weeks of gestation (Hustin and Schaaps, 1987). The use of Doppler ultrasound during pregnancy confirmed that there is very little blood flow within the IVS until 11–12 weeks of gestation (Foidart *et al*., 1992; Coppens *et al*., 1996) due to CTBs initially plugging the maternal spiral arterioles. This is consistent with acid-base and respiratory gas values in the fetal circulation and placental tissue during the first trimester (Jauniaux *et al*., 2001). At about 11 weeks of gestation the arteriole plugs are displaced and blood flow commences (Hustin and Schaaps, 1987; Burton *et al*., 1999). The onset of maternal blood flow is progressive, it starts at the periphery of the placenta and gradually extends towards the centre (Jauniaux *et al*., 2003a, b). As a result, the oxygen tension within the IVS rises from 18 mmHg (2.5%) at 8 weeks to ~60 mmHg (8.5%) at 12 weeks (Rodesch *et al*., 1992; Jauniaux *et al*., 2000; Burton and Caniggia, 2001), resulting in a burst of oxidative stress in the placenta, particularly in the syncytiotrophoblast (STB) (Jauniaux *et al*., 2000). This switch to a well-oxygenated environment serves a physiological role in stimulating trophoblast differentiation. However, early onset of maternal blood flow into the IVS results in a premature increase in placental oxidative stress (Jauniaux *et al*., 2003a, b) and is associated with early pregnancy loss (Hustin *et al*., 1990; Jauniaux *et al*., 1994; Jauniaux *et al*., 2003a, b). This premature onset of blood flow occurs prior to the establishment of robust mechanisms to ameliorate oxidative stress since the placenta only contains low levels of antioxidant enzymes at 8–10 weeks of gestation (Watson *et al*., 1997; Watson *et al*., 1998). Therefore, adequate trophoblast invasion and 'plugging' of the uterine arterioles resulting in a low oxygen environment in early pregnancy are essential for normal embryonic and placental development.

The above data suggest that trophoblast invasion early in pregnancy determines placental oxygenation. The question then is what causes inadequate trophoblast invasion in pathological pregnancies? Indeed there are many potential regulators of trophoblast invasion including trophoblast genotype, various growth factors and cytokines and interactions with maternal immune cells. Perhaps to some extent it is also true that the oxygen environment determines optimal trophoblast invasion. Oxygen concentration is a major influence on the balance

between CTB proliferation and invasion in cultured explants of early gestation human placentas.

Culturing 5–8-week-old placental villous explants in a low oxygen environment (2 or 3% O_2) stimulates EVT outgrowth associated with increased trophoblast proliferation compared with explants in a well-oxygenated environment (20% O_2) (Genbacev *et al*., 1997; Caniggia *et al*., 2000a, b). It has therefore been proposed that early in the first trimester (prior to 8 weeks) a low oxygen environment causes CTBs to proliferate but prevents their differentiation along the invasive pathway (Genbacev *et al*., 1997; Caniggia *et al*., 2000a, b; Caniggia and Winter, 2002). Similar conclusions were drawn from using villous explants from 8 to 14 weeks gestation cultured at 2–3, 8 or 20% oxygen (Genbacev *et al*., 1996; Seeho *et al*., 2008). In contrast, culture of placental samples from 8–10, 12–14 to 16–18 weeks gestation in 3% O_2 inhibited EVT invasion, with no change in cell proliferation, and increased apoptosis at 3 and 8% oxygen compared with 20% O_2 (Lash *et al*., 2006). Similarly, culture of 8–12 week villous explants in 1.5% oxygen resulted in fewer explants producing EVT outgrowths than those in 8% O_2 and a decrease in the area of outgrowth and number of cells that these explants produced (James *et al*., 2006).

Utilizing a different *in vitro* technique involving culture of myometrial spiral arteries with fluorescently labelled EVTs, culture in 3% oxygen suppressed EVT invasion regardless of the route (interstitial or endovascular) compared with those cultured in 17% oxygen (Crocker *et al*., 2005). However, there was more endovascular EVT invasion than interstitial invasion in 17% oxygen. These studies demonstrate conflicting data on the role of oxygen in the regulation of trophoblast function particularly regarding trophoblast proliferation, apoptosis and invasion. This may result from differences in media composition, the substrate the samples are grown on, oxygen levels used in the experiment or the gestational age of the trophoblast. In the future we would recommend that at a minimum, researchers include a group exposed to 5–10% oxygen in all studies. This is physiological for the placenta from 10 to 12 weeks onwards and will make results from different studies easier to interpret. Having only a control group exposed to 20% oxygen, which although a standard culture condition, is actually hyperoxic, and comparing this to a low oxygen tension, such as 2%, is only relevant when comparing results with the literature and makes data much more difficult to interpret and relate to the *in vivo* situation.

Furthermore, some studies suggest that culture of HTR-8/SVneo cells (a human first trimester cytotrophoblast cell line) in low (1%) oxygen conditions promotes invasion of these cells when compared with culture under standard conditions (20% oxygen) (Graham *et al*., 1998). In contrast, there has also been a report that culture of HTR-8/SVneo cells in 2% oxygen decreases trophoblast invasion and increases cell proliferation compared with those cultured in 20% oxygen (Kilburn *et al*., 2000). In a similar study, the invasiveness of HTR-8/SVneo cells was increased after 24 h but inhibited by 72 h in culture (Lash *et al*., 2007). The presence of serum, the addition of plasminogen or other factors, the reduced oxygen levels, or the use of a different invasion assay in the Graham *et al*. (1998) study may account for the different results observed. Microarray and real-time RT–PCR analyses of HTR-8/SVneo cells exposed to either 20 or 1% oxygen have revealed several genes that encode proteins involved in cell migration, angiogenesis and apoptosis, as well as anti-invasive factors which are up-regulated in low oxygen (Koklanaris *et al*., 2006), suggesting that a low oxygen environment may 'fine-tune' trophoblast differentiation.

Importantly, it is difficult to relate these *in vitro* observations to the situation that occurs *in vivo*, especially when there is such an array of conflicting evidence in the literature. It is clear that *in vivo*, invading trophoblast cells are exposed to an oxygen gradient as they migrate from the trophoblast cell column into the maternal decidua. Invading CTBs respond to this increased oxygen availability by altering the expression of various cell adhesion molecules and proteases, promoting further migration. We suggest that future research efforts are directed toward assessing whether these current *in vitro* models truly reflect the environment *in vivo*, by matching gestational age and oxygen concentration as much as possible. Additionally, a much more detailed characterization of placental development as it occurs *in vivo* is required (including trophoblast differentiation, invasion, proliferation and vasculogenesis) as well as of the molecules involved in the regulation of these events and their interaction (e.g. growth factors, cytokines, oxygen levels). Of course this has proven difficult, since there is a lack of early gestation samples for which the pregnancy outcome is known.

Placental hypoxia late in pregnancy

Insufficient trophoblast invasion and defective vascular remodelling of the uterine spiral arteries in the first trimester can lead to increased rate of blood flow, which damages the villous architecture, and an increased risk of spontaneous vasoconstriction and ischemia-reperfusion injury resulting in oxidative stress (Burton *et al*., 2009). This causes impaired placental perfusion and hence has been assumed to result in chronic placental ischemia and hypoxia later in gestation, which is detrimental to pregnancy success and may lead to pre-eclampsia and/or IUGR, and in some cases will result in preterm delivery (Chaddha *et al*., 2004; Viero *et al*., 2004). Although the preeclamptic and IUGR placentas are thought to be hypoxic, to date there have been no direct measurements to support this assumption. Interestingly, in pregnancies at high altitude, where placental hypoxia has been considered to be exacerbated, infant birthweight is decreased, primarily due to a slowing of fetal growth in the third trimester (reviewed in Zamudio, 2003; Moore *et al*., 2004), and these pregnancies have an increased incidence of pre-eclampsia. Subsequently, there is also an increased incidence of perinatal and infant mortality and morbidity in pregnancies at high altitude. Importantly, placentas from uncomplicated pregnancies at high altitude show no indication of significant hypoxic stress at term compared with placentas from pregnancies at low altitude and appear to have successfully adapted to the lower oxygen atmosphere (Tissot van Patot *et al*., 2004; Zamudio *et al*., 2007a, b). It is unclear then whether these placentas can actually be considered hypoxic and it will be important in future to determine why some placentas apparently adapt to the hypoxic environment and others do not.

Thus, oxygen appears to play a critical role in the development of pregnancy complications and is a key mediator of placental development and function. During pregnancy a low oxygen environment can result in different outcomes depending on the timing of exposure in pregnancy, the severity of the hypoxic exposure and the duration. The exact mechanism by which trophoblasts sense oxygen tension is currently unclear. However, several potential pathways have been

identified: these pathways often utilize redox-sensitive transcription factors, of which the HIF family is the best characterized in trophoblasts.

Expression and function of HIFs in the placenta

Both HIF-1α and HIF-2α proteins are constitutively expressed in the human placenta. In the first trimester, HIF-1α and -2α have been localized in the STB, villous CTB and fetoplacental vascular endothelium (Rajakumar and Conrad, 2000; Genbacev *et al*., 2001), consistent with the low oxygen environment of the placenta during the first trimester of pregnancy, but their abundance decreases significantly with gestational age (Caniggia *et al*., 2000a, b; Rajakumar and Conrad, 2000; Caniggia and Winter, 2002). More specifically Ietta *et al*. (2006) have described in detail the dynamic expression of HIF and HIF regulators during placental development. HIF-1α and VHL mRNA and protein are present throughout gestation, with levels peaking at 7–10 weeks of gestation, when the oxygen tension is low, and declining thereafter (Ietta *et al*., 2006).

During early pregnancy, HIF-1α can be immunolocalized to CTBs and EVTs while VHL is present in STB. By 10–12 weeks, HIF-1α disappears, coinciding with an increase in expression of VHL in the CTB and an increase in VHL:HIF-1α binding throughout the placenta (Ietta *et al*., 2006). The expression of ARNT and HIF-2α mRNA and protein are present and stable throughout gestation (Ietta *et al*., 2006). The PHD enzymes (PHD1, 2, 3) are also dynamically expressed in an oxygen dependent fashion, peaking at 10–12 weeks gestation (Ietta *et al*., 2006). Previous reports by others have found slightly different results. In 2000, Rajakumar *et al*. reported that the levels of *Hif-1α* mRNA in the placenta do not change across gestation, while *Hif-2α* mRNA abundance increases as gestation proceeds (Rajakumar and Conrad, 2000). However, it would be most pertinent to quantify HIF-1α and HIF-2α proteins and determine their cytoplasmic versus nuclear localization and DNA binding in the placenta across gestation in order to understand HIF-1 activity.

In a preliminary report examining pregnancies at high altitude, HIF-1 DNA binding (measured by enzyme-linked immunosorbent assay) and HIF-associated proteins were unchanged compared with placentae from low altitude pregnancies (Tissot van Patot *et al*., 2004), leading to the conclusion that there was placental adaption to the hypoxic environment. However, in a more recent and comprehensive study, HIF-1α and VHL mRNA and protein levels were significantly increased in placentae from high altitude pregnancies, whereas *Hif-2α* and *Arnt* levels are unchanged (Zamudio *et al*., 2007a, b). Similarly, HIF-1α and HIF-2α are over-expressed in the villous placenta of women with pre-eclampsia compared with normal term pregnancies (Rajakumar *et al*., 2001; Rajakumar *et al*., 2004), with the expression of HIF-1α in villous and extravillous trophoblast cells being similar to the expression in trophoblasts prior to exposure to increased oxygen in first trimester (Caniggia and Winter, 2002). Oxygen-dependent down-regulation of HIF-1α and -2α protein is impaired in pre-eclampsia (Rajakumar *et al*., 2003), likely a result of both increased formation and decreased degradation owing to proteosome dysfunction (Rajakumar *et al*., 2008). Consistent with this, others have reported enhanced placental expression of HIF target genes, including *Vegf*, in pregnancies complicated by pre-eclampsia and IUGR (Helske *et al*., 2001), which maybe suggestive of a vasculo-adaptative response to chronic hypoxia at the fetoplacental–maternal interface. Both HIF-1α and HIF-2α are therefore in an ideal position to regulate placental development throughout gestation in both normal and pathological pregnancies.

Differential regulation of HIF-1α and HIF-2α in acute versus prolonged hypoxia

The degradation of the HIF-α protein and its stabilization under hypoxia has been the focus of much research in the last 10 years. In particular, it is apparent that HIF-1α and HIF-2α may respond differently depending on the length and severity of hypoxic exposure. HIF-2α is expressed to a higher degree than HIF-1α in a number of endothelial, fibroblast-like and epithelial cell lines in normoxic conditions (Wiesener *et al*., 1998). In addition, HIF-2α is increased to a greater extent than HIF-1α following exposure to mild hypoxia (5% O_2) (Wiesener *et al*., 1998; Holmquist-Mengelbier *et al*., 2006). It has now been proposed that HIF-1α stabilization in hypoxia (1% O_2) is an acute response, which is diminished under prolonged periods of low oxygen exposure (reviewed in Lofstedt *et al*., 2007). Conversely, HIF-2α protein levels are increased under mild hypoxia (5% O_2) and continue to increase with time, thereby playing an important role in mediating effects on gene expression under conditions of prolonged exposure to low oxygen. In addition, it is likely that a different set of HIF targets are up-regulated in response to acute versus prolonged hypoxia but this phenomenon is yet to be examined in the placenta.

Differential regulation of the mRNA for the α subunits has been reported in response to acute and chronic hypoxia. For instance, in rats, *Hif-3α*, but not *Hif-1α* or *Hif-2α*, mRNA levels increase in the cerebral cortex, hippocampus, lung, heart, liver and kidney after 2 h of *in vivo* hypoxic exposure but no change was seen within 30 min (Heidbreder *et al*., 2003). In contrast, a previous study had shown that *Hif-1α* mRNA in rat and mouse brain, kidney and lung was up-regulated within 30 min of hypoxia treatment *in vivo*, but returned to baseline after 4 h (Wiener *et al*., 1996). Similarly, Wang *et al*. showed that hypoxia induces the expression of *Hif-1* mRNA *in vitro* but this is diminished by longer periods of hypoxia (Wang *et al*., 1995; Uchida *et al*., 2004). These studies suggest that *Hif-1α* mRNA is transiently increased in response to hypoxic stimuli. Consistent with this, others have reported unaltered *Hif-1α* mRNA levels in mice in response to 4 h of hypoxia *in vivo* (Wenger *et al*., 1996).

In 1999, a naturally occurring antisense transcript of HIF-1α (*asHif-1α*) was discovered in human renal cancer (Thrash-Bingham and Tartof, 1999). More recently, *asHif-1α* was identified in the kidney, liver, testis, brain, lung, spleen, heart and skeletal muscle of rodents (Rossignol *et al*., 2004) and specifically in the mouse decidua and trophoblasts early in gestation (Pringle *et al*., 2007). It has been suggested that asHIF-1α exposes AU rich elements in the 3′untranslated region of *Hif-1α* mRNA which decreases the stability of *Hif-1α* mRNA (Fig. 1), impairing HIF-1α protein translation and expression (Rossignol *et al*., 2002). Conversely, *Hif-2α* mRNA is not destabilized by asHIF-1α owing to the lack of AU rich elements (Uchida *et al*., 2004). In fact, *Hif-2α* transcription can

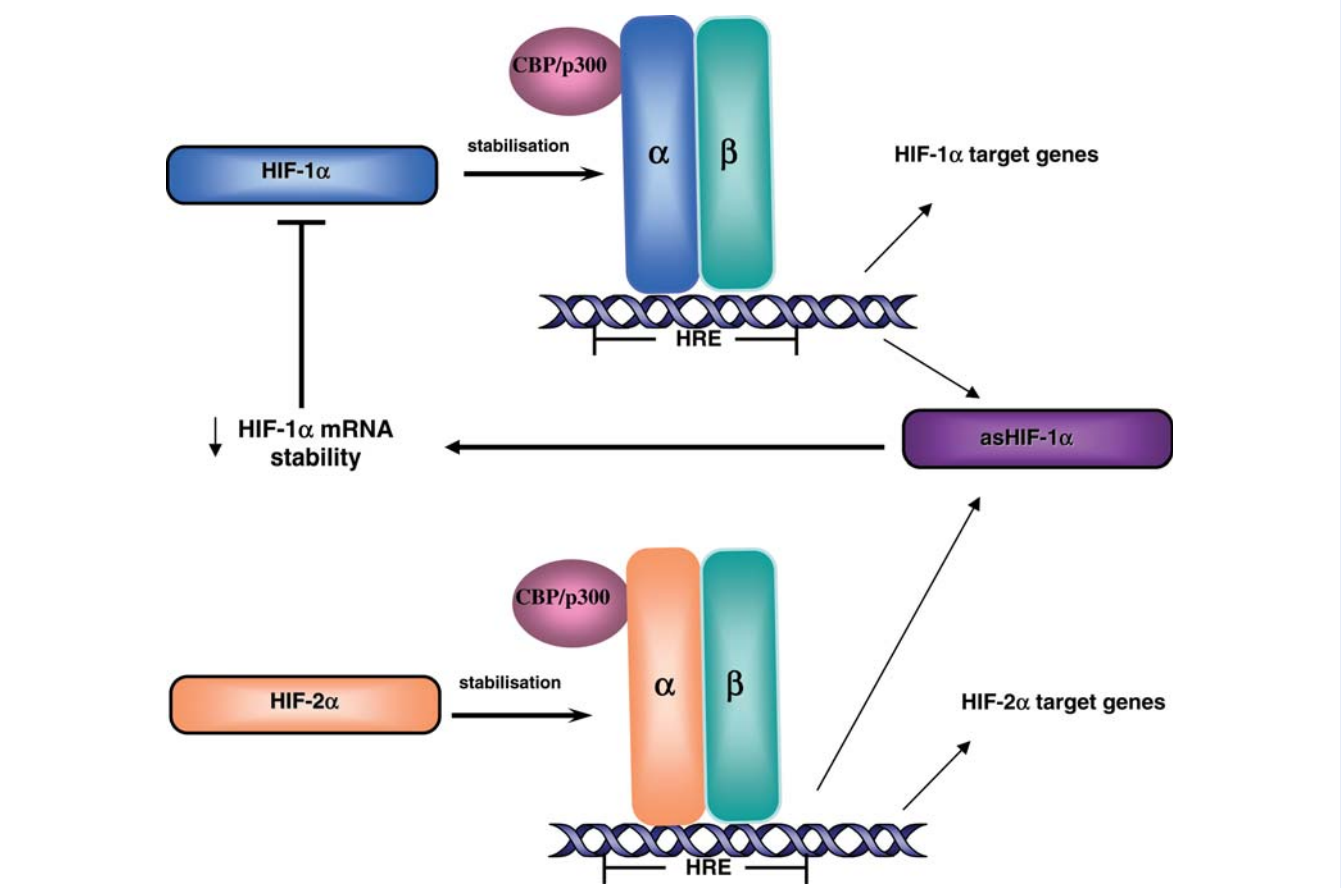

Figure 1 Mechanism of HIF regulation under acute and prolonged hypoxia. Under acute hypoxia, HIF-1α and HIF-2α are stabilized and can induce the transcription of their target genes. Following exposure to prolonged hypoxia, HIF-1α and HIF-2α can up-regulate the expression of the antisense transcript of HIF-1α (asHIF-1α) which, in turn, destabilizes HIF-1α mRNA thereby reducing HIF-1α protein levels and HIF-1α mediated gene transcription. Therefore under prolonged hypoxia HIF-2α mediated transcription is more abundant. HRE: hypoxia response element.

increase in hypoxia when it increases its own promoter activity (Sato *et al.*, 2002). Since *asHif-1α* contains a putative HRE it is assumed that both HIF-1 and HIF-2 are involved in the regulation of *asHif-1α* expression thus creating a negative feedback loop of HIF regulation under prolonged hypoxia (Fig. 1) (Rossignol *et al.*, 2002; Uchida *et al.*, 2004).

In the placenta, some evidence of differing responses to acute versus prolonged hypoxic exposure can be found but to date no studies have examined this directly. Human placental villous explants from 5 to 8 weeks gestation, cultured at 3% oxygen demonstrated significantly greater mRNA expression of *Hif-1α* when compared with explants cultured at 20% oxygen, after 24 h of culture (Caniggia *et al.*, 2000a, b). First trimester or term villous explants exposed to 2% oxygen for 3–24 h showed increases in HIF-1α and HIF-2α protein expression, with no change in their mRNA levels, and increased HIF-1 DNA binding activity (Rajakumar and Conrad, 2000). However, in cultured 6–8 week placenta it was HIF-2α protein that dramatically increased following 48 h exposure to 2% oxygen, whereas HIF-1α levels were the same in those explants cultured under hypoxic and normoxic conditions (Genbacev *et al.*, 2001). These *in vitro* studies have yielded inconclusive results, which may result from the use of different oxygen tensions, culture conditions or duration of exposure.

In the future it will be important to investigate the effect of different oxygen concentrations and prolonged versus acute hypoxic exposure on HIF mRNA and protein and their targets throughout placental development in more detail. In addition, asHIF-1α expression and localization in the human placenta has yet to be elucidated. In the mouse, we have found that culture of early trophoblasts in 1% oxygen for 3 days significantly decreases *Hif-2α*mRNA abundance whereas *Hif-1α*mRNA levels remain unchanged (Pringle *et al.*, 2007). Furthermore, our data suggest that the differential effects on *Hif-1* and *2α* mRNA levels may be mediated by an increase in *asHif-1α* mRNA in trophoblasts cultured in 1% oxygen. Research in this area may be of critical importance when considering the role of HIFs in prolonged hypoxic exposure of the placenta, such as occurs in pre-eclampsia and pregnancy at high altitude.

Non-hypoxic induction of HIF-1α

HIF-1α can also be induced by factors other than hypoxia, including hormones, cytokines and growth factors (Table 1), many of which are expressed in the placenta or are increased in the maternal

Table 1 Non-hypoxic regulators of HIFs.

Non-hypoxic stimuli	Action	Proposed mechanism	Found in placenta (Yes/No)	HIF target gene (Yes/No)	References
Progesterone	Increased *Hif-1α* mRNA, or HIF-1α and HIF-2α protein	?	Yes	No	Daikoku *et al.* (2003), Song *et al.* (2008)
Estrogen	Increased *Hif-2α* mRNA	?	Yes	No	Daikoku *et al.* (2003)
Prostaglandin E2	Increased HIF-1α protein	ERK, AKT, C-SRC tyrosine kinase activity	Yes	No	Fukuda *et al.* (2003)
Angiotensin II	Increased HIF-1α mRNA and protein	AT1R, PKC, ROS activation	Yes	No	Richard *et al.* (2000), Page *et al.* (2002), Araki-Taguchi *et al.* (2008)
Thrombin	Increased HIF-1α mRNA, protein and activity	AT1R, PKC, ROS activation	Yes	No	Richard *et al.* (2000), Gorlach *et al.* (2001), Page *et al.* (2002)
Platelet derived growth factor	Increased HIF-1α protein and activity	Via ROS activation	Yes	No	Richard *et al.* (2000), Gorlach *et al.* (2001)
Transforming growth factor-β1	Increased HIF-1α protein via inhibiting HIF-1α degradation	Decreased VHL mRNA and protein via Smad pathway	Yes	Yes	Gorlach *et al.* (2001), McMahon *et al.* (2006)
Endothelin-1	Increased HIF-1α protein	?	Yes	Yes	Spinella *et al.* (2002)
Epidermal Growth Factor	Increased HIF-1α protein	Via PI3K	Yes	No	Jiang *et al.* (2001)
RhoA	Increased HIF-1α protein	?	Yes	No	Hayashi *et al.* (2005)
Mechanical stretch	Increased HIF-1α mRNA and protein	Via p42/p44 MAPK pathway			Chang *et al.* (2003)
Interleukin-1β	Increased HIF-1 protein	ERK 1/2 activation	Yes	Yes	Hellwig-Burgel *et al.* (1999), Karmakar and Das (2002), Qian *et al.* (2004)
Tumor Necrosis Factor-α	Increased HIF-1 DNA binding	Activation of HIF co-activators, NFκB pathway	Yes	No	Zhou and Bondy (1992), Hellwig-Burgel *et al.* (1999), Huber *et al.* (2006)
Insulin	Increased *Hif-3α* mRNA, HIF-1α and HIF-3α protein	Via PI3 and MAPK pathways	Yes	No	Zelzer *et al.* (1998), Feldser *et al.* (1999), Jiang *et al.* (2001), Heidbreder *et al.* (2003), Treins *et al.* (2002)
Insulin-like Growth Factor-I	Increased HIF-1α and HIF-2α protein	Via PI3 and MAPK pathways or through PHD2	Yes	No	Zelzer *et al.* (1998), Feldser *et al.* (1999), Chavez and LaManna (2002), Richard *et al.* (2000)
Insulin-like Growth Factor-II	Increased HIF-1α protein, decreased *Hif-2α* mRNA	Via Mdm2 and decreased p53	Yes	Yes	Feldser *et al.* (1999), Kwon *et al.* (2004), Pringle *et al.* (2007)

circulation during pregnancy. Indeed, HIF-1α protein is up-regulated in a well-oxygenated environment (~20% oxygen) during Matrigel induced endovascular differentiation in a human EVT cell line (Fukushima *et al.*, 2008). The mechanisms, however, are unknown.

Hif-1α mRNA can be regulated by progesterone in the mouse uterus whereas estrogen has the ability to regulate *Hif-2α* mRNA (Daikoku *et al.*, 2003). In sheep, progesterone treatment increases HIF-1α and HIF-2α protein but not mRNA in endometrial luminal and superficial glandular epithelium (Song *et al.*, 2008). Similarly, exposure of colon cancer cells to prostaglandin E2 (PGE2) induces the expression of vascular endothelial growth factor (VEGF) via increased HIF-1α (Fukuda *et al.*, 2003). HIF-1α protein levels in vascular smooth muscle cells are strongly increased in normal oxygen conditions when cells are stimulated with angiotensin II (Ang II), thrombin, platelet derived growth factor (PDGF) or Transforming Growth Factor-β1 (TGF-β1) (Richard *et al.*, 2000; Gorlach *et al.*, 2001; Page *et al.*, 2002). More recently, it was found that TGF-β1 decreases mRNA and protein levels of PHD2, through the Smad signalling pathway, thereby inhibiting the degradation of HIF-1α (McMahon *et al.*, 2006). Other non-hypoxic inducers of HIF-1α include Endothelin 1 (Spinella *et al.*, 2002), epidermal growth factor (Jiang *et al.*, 2001), Rho A which regulates actin stress fibres (Hayashi *et al.*, 2005) and even cyclical mechanical stretch (Chang *et al.*, 2003).

Of particular interest is a recent study demonstrating that in first trimester placental villous explants Ang II mimics the effects of hypoxia

by increasing HIF-1α mRNA and protein (Araki-Taguchi *et al*., 2008). In addition, Ang II increased EVT outgrowth and the number of cells in CTB cell columns and increased Ki67 suggesting an increase in proliferation (Araki-Taguchi *et al*., 2008). Ang II has also been shown to increase plasminogen activator inhibitor (PAI)-1 synthesis and secretion in human trophoblasts through the type 1 angiotensin receptor (AT_1R) and is associated with decreased trophoblast invasion (Xia *et al*., 2002; Araki-Taguchi *et al*., 2008). Interestingly, it has been proposed that Ang II and the renin angiotensin system may play a role in the development of pre-eclampsia. Ang II levels in the placental bed are significantly elevated in women with pre-eclampsia (Anton *et al*., 2009), and may act on the placental villi, which display an up-regulation of AT_1Rs (Anton *et al*., 2008),

Cytokines also regulate HIF in normoxia. For example, interleukin (IL)-1β and tumour necrosis factor (TNF)-α increased HIF-1 DNA binding in human hepatoma cells (Hellwig-Burgel *et al*., 1999). IL-1β increased HIF-1α nuclear protein, whereas the positive effect of TNF-α on HIF-1 DNA binding was suggested to result from activation of HIF co-activator proteins (Hellwig-Burgel *et al*., 1999). Similarly, a more recent study found that IL-1β induces HIF-1α-mediated VEGF secretion in normal human trophoblast cells and this may be a result of ERK 1/2 activation (Qian *et al*., 2004). Interestingly, matrix metalloproteases (MMP-2, MMP-9) and urokinase plasminogen activator (uPA), which are abundantly expressed by CTBs and degrade the extracellular matrix (ECM), were also up-regulated by IL-1β in human trophoblasts (Karmakar and Das, 2002). In contrast, HIF-1α protein accumulation induced by TNFα requires the NFκB pathway (Zhou *et al*., 2003). In addition, TNFα inhibits trophoblast migration through elevation of PAI-1 in first trimester villous explant cultures (Bauer *et al*., 2004; Huber *et al*., 2006) likely by activation of NFκB (Huber *et al*., 2006). Indeed a recent review by Redman and Sargent (2009) emphasizes the role of inflammatory stimuli in HIF accumulation. This may be important in both normal pregnancies, where a systemic inflammatory response is evident, and in pathological pregnancies, such as pre-eclampsia, where inflammation is exaggerated. To date there have been few studies on the role of HIF in the placental inflammatory response but research in this area may provide novel insights in the future.

Although insulin-like growth factor (IGF)-II is known to be a target gene of HIF-1 (Feldser *et al*., 1999), insulin, IGF-I and IGF-II are all able to induce HIF-1α protein expression in various cell lines (Zelzer *et al*., 1998; Feldser *et al*., 1999; Jiang *et al*., 2001; Chavez and LaManna, 2002; Treins *et al*., 2002; Thomas and Kim, 2008; Alam *et al*., 2009). Similarly, both insulin and 2-deoxy-D-glucose treatment resulted in a widespread increase in HIF-3α mRNA and protein (Heidbreder *et al*., 2007). IGF-I can also induce VEGF expression in human osteoblast-like cells through transcriptional activation involving the HIF-2α/ARNT complex (Akeno *et al*., 2002). However, the magnitude of the increase in HIF protein induced by insulin or IGFs *in vitro* is far less than that caused by hypoxia. In contrast, IGF-II treatment of mouse trophoblasts *in vitro* has been shown to decrease *Hif-2α*, *asHif-1α*, *Glut-1* and *Vegf* mRNA levels following culture in prolonged hypoxia, but not in well oxygenated cells (Pringle *et al*., 2007). This suggests that the response to growth factors varies depending on the oxygen availability.

Many, if not all, of the non-hypoxic regulators of HIF protein abundance reported to date are present and active in the placenta (Table I). To date, the role of HIF in mediating their effects in the placenta has been little studied. As an initial step, it will be important in the future to identify which of these non-hypoxic regulators of HIF (outlined in Table I) are playing a similar role in the placenta. From there we can start to examine the role of non-hypoxic HIF regulation in placental development and when this may occur. This would be a novel approach to research on placental HIF and will provide exciting insights into the regulation of placental development. In addition, it is important to consider possible interactions between hypoxic and non-hypoxic HIF regulators and the effects these may have. Importantly, it will be essential to also examine the role of both hypoxic and non-hypoxic HIF regulation in pathological pregnancies, such as pre-eclampsia, and whether these interact.

Regulation of placental development and function by HIF target genes

HIF target genes may therefore be regulated by either hypoxic or non-hypoxic stimuli and many of these genes have critical roles in placental development and function. Interestingly, some HIF target genes, such as IGF-II and TGF-β, are also non-hypoxic regulators of HIF (Table I) whereas others can feed back to regulate HIF in prolonged hypoxia. Therefore, these molecules may provide novel co-regulatory responses or feedback pathways in the regulation of placental development. Many other HIF target genes have not yet been investigated in this way but further research may provide interesting insights into the complexity of interrelationships between HIF and its target genes. Here, we outline just some of the numerous roles that HIF-regulated genes have in placental development and how they could interact to regulate its differentiation, growth and function through HIF dependent or independent mechanisms.

Trophoblast invasion and migration

Hif1α −/− Hif2α −/− mice display a 17% reduction in trophoblast invasion compared with wild type placentae (Cowden Dahl *et al*., 2005). Several pro- and anti-invasive factors that are expressed by the trophoblast cells themselves or by the maternal decidua are HIF target genes. Furthermore, these factors seem to interact in such a way that they are able to tightly coordinate the extent and timing of trophoblast invasion and migration.

uPA system

uPA, the uPA receptor (uPAR) and uPA inhibitors play a central role in trophoblast migration and invasion. EVTs have been shown to exhibit a highly polarised distribution of uPAR-bound uPA at the invasive front (Multhaupt *et al*., 1994). uPA is secreted as an inactive proenzyme (pro-uPA) which, upon binding to its specific uPAR on the cell surface, is cleaved to its active form. Following activation, cell membrane bound uPA can catalyse the conversion of the inactive zymogen plasminogen, to the active proteinase plasmin (Kjoller *et al*., 1997) which can then go on to activate MMPs and directly degrade certain ECM components required for invasion and remodelling of the maternal decidua. This process is modulated by tissue inhibitors of matrix metalloproteinases (TIMPs) and PAI-1 which are predominantly synthesized by the decidua but also by the invading

trophoblasts (Yebra *et al*., 1996; Floridon *et al*., 2000). Interestingly, the uPA:uPAR interaction can stimulate EVT migration independently of uPA catalytic action, via the mitogen activated protein kinase (MAPK) pathway and calcium signalling events that require phosphatidylinositol-3 kinase and phospholipase C (Liu *et al*., 2003).

Culture of HTR-8/SVneo trophoblasts less than 1% oxygen results in elevated expression of uPAR at the mRNA and cell surface bound protein levels and promotes trophoblast invasion (Graham *et al*., 1998). However, a more recent study has shown that culture in 3% oxygen inhibits early gestation EVT invasion, increases uPAR and PAI-2 expression and decreases uPAR activity (Lash *et al*., 2006). Although direct experimental evidence for HIF regulation of uPA in placenta is currently lacking, PAI-1 mRNA and protein levels have been shown to increase in HTR-8/SVneo cells in response to hypoxia via HIF-1α or HIF-2α [determined by small interfering RNA (siRNA) against HIF-1α and -2α] (Fitzpatrick and Graham, 1998; Meade *et al*., 2007) and may provide a mechanism for the control of trophoblast invasion.

Insulin-like growth factor-II

IGF-II is regulated by HIF (Feldser *et al*., 1999) among other factors and is thought to play an important role in placental development. Maternal circulating concentrations of IGF-II increase during early pregnancy in humans (Wilson *et al*., 1982; Gargosky *et al*., 1990). In addition, presumed *in vivo* hypoxia at term has been associated with increased levels of placental *Igf2* mRNA (Trollmann *et al*., 2007) whereas placental IGF-II protein is elevated in preeclamptic placenta (Gratton *et al*., 2002). Direct evidence for HIF regulation of IGF-II in the placenta has not yet been reported but warrants further investigation. Indeed, IGF-II has been shown to promote migration of human EVT cells (Irving and Lala, 1995; Hamilton *et al*., 1998; McKinnon *et al*., 2001), increase proliferation in first trimester villous CTBs *in vitro* (Hills *et al*., 2004; Forbes *et al*., 2008) and decrease CTB apoptosis (Forbes *et al*., 2008). IGF-II induced migration of EVT cells *in vitro* is thought to be mediated by the IGF2R using a Gi-coupled receptor and (MAPK) intracellular signalling pathway (McKinnon *et al*., 2001).

The bioavailability and biological actions of the IGFs are regulated by a family of 6 IGF binding proteins (IGFBPs 1-6). Interestingly, IGFBP-1, -2 and -3 are also HIF regulated genes (Tazuke *et al*., 1998; Feldser *et al*., 1999). In blood, ~75% of IGFs circulate as a complex with IGFBP-3 or IGFBP-5 and the acid-labile subunit, which acts as a reservoir (Mohan and Baylink, 2002). The remaining IGFs in the circulation bind to one of the remaining IGFBPs enabling them to cross the endothelium, increasing their availability to local tissues. Binding of IGFs to IGFBPs can result in either inhibition or enhancement of IGF actions. The affinity of IGFBPs for IGFs is higher than that of the receptors for IGFs, therefore binding of IGFBPs to IGFs prevents interactions with the IGF receptors (Clemmons, 1998; Baxter, 2000; Mohan and Baylink, 2002; Rosenzweig, 2004; Duan and Xu, 2005). Conversely, several studies have also shown that a number of IGFBPs are able to stimulate IGF actions in a variety of cell types. Indeed, the same IGFBP can act to enhance or inhibit IGF actions depending on the cell type, abundance of IGFBP and post-translational modifications such as glycosylation or phosphorylation, which result in altered affinity for IGFs (Baxter, 2000; Mohan and Baylink, 2002; Duan and Xu, 2005). Furthermore, IGF-II can alter the phosphorylation state of IGFBP-1 at the feto-maternal interface promoting its own actions (Westwood *et al*., 1997). Several IGFBPs are also associated with the cell surface or ECM, reducing their affinity for the IGFs, and hence can also modulate IGF actions (Clemmons, 1998; Baxter, 2000; Mohan and Baylink, 2002).

Transforming growth factor-β

The TGF-β isoforms are believed to be major factors regulating CTB behaviour and are commonly thought to inhibit cell proliferation and invasion (Bischof, 2001). TGFβ3 expression is induced by hypoxia both *in vivo* and *in vitro* and is directly regulated by HIF-1 in trophoblast cells (determined by siRNA knockdown of HIF-1α) (Caniggia *et al*., 2000a, b; Schaffer *et al*., 2003; Nishi *et al*., 2004). Interestingly, both HIF-1α and TGF-β3 are overexpressed in preeclamptic placentae (Nishi *et al*., 2004). However, during normal pregnancy, the decidua is the major source of TGF-β. TGF-β1 inhibits EVT invasion via the RhoA/Rho-associated kinase (ROCK) pathway (Fafet *et al*., 2008) by up-regulating integrin expression (Irving and Lala, 1995) and reducing uPA activity *in vitro* via decreasing the secretion of uPA and increasing the production and secretion of PAI-1 (Graham, 1997; Fitzpatrick and Graham, 1998). Similarly, TGF-β1 can up-regulate TIMP-1 and -2, PAI-1 and -2 (Karmakar and Das, 2002) and deposition of ECM components (Feinberg *et al*., 1994) and adhesive cell surface molecules (Meisser *et al*., 1999; Karmakar and Das, 2004) in human trophoblasts *in vitro*. TGF-β1 is also known to inhibit steroidogenesis in human trophoblasts (Luo *et al*., 2002) and promotes syncytium formation in isolated first trimester trophoblast primary cultures by increasing surface expression of cadherin-11 (Getsios *et al*., 1998). In contrast to this, first trimester villous explants, exposed to either antisense TGF-β3 oligonucleotide or TGF-β3 antibody (but not those for TGF-β1 or TGF-β2), displayed prominent EVT outgrowth from the distal end of the villous tip and an increased number of cells migrating into the surrounding matrix (Caniggia *et al*., 1999).

TGF-β is secreted in a latent pro-form in complex with a latency associated peptide (LAP) and has the ability to bind the IGF2 receptor (IGF2R) (via M6P residues in the LAP) which interacts with the uPAR on the cell surface (Godar *et al*., 1999). uPA binding to uPAR allows uPA to cleave plasminogen into plasmin which can then go on to cleave the LAP, and active TGF-β1 is released. This provides a novel interaction between the uPA, IGF-II, TGF-β and HIF systems whereby, depending on the relative abundance of IGF-II and/or TGF-β, the uPAR/IGF2R interaction can be utilized to either promote or inhibit trophoblast migration and invasion. Importantly, hypoxia-induced HIF regulation of IGF-II, TGF-β, PAI-1, uPAR and uPA will also impact on this system and ultimately, trophoblast behaviour. In addition, non-hypoxic regulation of HIFs by IGF-II and TGF-β may provide additional feedback pathways.

Placental vascularization

It is thought that the low oxygen environment in the placenta controls CTB vascular remodelling and acts as a major regulator of placental angiogenesis. Indeed, *Hif1α −/− Hif2α −/−* mice have impaired placental vascularization (Cowden Dahl *et al*., 2005). The VEGFs are key molecules involved in angiogenesis. The VEGF family consists of VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF), the latter of which is closely related to

VEGF-A. There are three VEGF family receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR, called Flk-1 in mice) and VEGFR-3, as well as a soluble form of Flt-1 (sFlt-1) that is generated by alternative splicing and lacks transmembrane and cytoplasmic domains (Kendall and Thomas, 1993). VEGF is thought to act locally via its receptors, VEGFR-1 and VEGFR-2, to establish the fetoplacental circulation. VEGF is hypoxia inducible and is a well-known HIF target gene (Wang and Semenza, 1995). Circulating sFlt-1 binds VEGF and PlGF with high affinity thereby decreasing their availability for the transmembrane receptors VEGFR-1 and VEGFR-2. Although not members of the VEGF family, the angiopoietins are also involved in placental vasculogenesis through their interaction with tyrosine kinase receptor (Tie-2) and are influenced by oxygen availability (Zhang et al., 2001). It appears that rather than solely their concentration at the feto-maternal interface, the ratio and interaction of VEGF family members, angiopoietins and growth factors orchestrate the development of the placental vasculature and all in all, this is influenced by oxygen (Charnock-Jones and Burton, 2000).

Placental villous and extravillous trophoblasts secrete sFlt-1 during pregnancy (Clark et al., 1998) and sFlt-1 levels in the maternal circulation increase with increasing gestational age (Koga et al., 2003). Interestingly, *sFlt-1* mRNA levels and sFlt-1 secretion into culture media are up-regulated following exposure to a low oxygen environment in villous explants from first trimester and uncomplicated and preeclamptic pregnancies at term (Rajakumar et al., 2009). HIF-1α knockdown using antisense oligonucleotides has demonstrated that this up-regulation is mediated by HIF-1α in first trimester villous explants (Nevo et al., 2006). In addition, placental sFlt-1 is up-regulated in pre-eclampsia, leading to increased maternal circulating concentrations of sFlt-1 that precede symptoms and fall after delivery (Koga et al., 2003; Maynard et al., 2003; Rajakumar et al., 2005; Rajakumar et al., 2007; Rajakumar et al., 2009).

Recently, multiple isoforms of sFlt-1 have been identified in the circulation of women with pre-eclampsia as well as in uncomplicated pregnancies (Rajakumar et al., 2009). *Vegf* and its receptor *Flt-1* mRNA levels are also increased in preeclamptic placenta compared with controls (Tsatsaris et al., 2003; Rajakumar et al., 2004; Rajakumar et al., 2005; Rajakumar et al., 2007). Conversely, membrane bound Flt-1 is decreased in the placental bed of patients with pre-eclampsia (Tsatsaris et al., 2003). Pre-eclampsia is associated with an increase in total circulating VEGF, but decreased free VEGF and PlGF a result of increased sFlt-1 (Maynard et al., 2003; Tsatsaris et al., 2003) and this is thought to contribute to endothelial dysfunction seen in pre-eclampsia. Indeed, administration of sFlt-1 to pregnant rats induces hypertension, proteinuria and glomerular endotheliosis (Maynard et al., 2003). Of considerable interest is the observation that experimental induction of utero-placental insufficiency (ischaemia with restricted transport function) by uterine artery ligation in baboons resulted in a pre-eclampsia-like syndrome with hypertension and proteinuria and, notably, elevated placental and maternal circulating monocyte*Flt-1* mRNA and increased sFlt-1 protein in plasma (Makris et al., 2007).

Recently, sFlt-1 was shown to be up-regulated in pregnant mice lacking catechol-*O*-methyltransferase (*Comt* −/− mice) (Kanasaki et al., 2008). COMT generates 2-methoxyoestradiol (2-ME) from hydroxyoestradiol, a potent inhibitor of angiogenesis (Fotsis et al., 1994; Mabjeesh et al., 2003). These *Comt* −/− pregnant mice display a pre-eclampsia-like phenotype, including endothelial damage in the decidual arteries and renal glomeruli, gestational hypertension and proteinuria (Kanasaki et al., 2008). Importantly, this pre-eclampsia-like phenotype is restored by the administration of 2-ME. This is in accordance with human preeclamptic pregnancies which display decreased levels of 2-ME and COMT (Kanasaki et al., 2008). Additionally, *Comt* −/− mice display signs of hypoxia (as determined by hypoxyprobe localization), elevated nuclear HIF-1α in the placenta and increased circulating sFlt-1, which was prevented by 2-ME administration. 2-ME is a negative regulator of HIF-1α (Mabjeesh et al., 2003) and was further shown to suppress hypoxia-induced HIF-1α and sFlt-1 in HTR-8 trophoblast cells (Kanasaki et al., 2008). This very elegant study by Kanasaki *et al.* provides very strong evidence that disruption of COMT/2-ME resulting in elevated HIF-1α and subsequent sFlt-1 leads to angiogenic dysfunction, placental insufficiency and ultimately, the preeclamptic phenotype in mice.

Placental transport

Glucose transport is up-regulated by hypoxia in a number of cell types and is crucial for fetoplacental growth. In term placental villous explants, *in vitro* hypoxic exposure increases *Glut1* and *Glut3* mRNA levels and significantly increases glucose consumption (Esterman et al., 1997). It was subsequently shown that hypoxic induction of *Glut1* mRNA and protein in human BeWo and rat Rcho-1 trophoblast cell lines was a result of HIF-1α interacting with a consensus HRE site in the *Glut-1* promoter (Hayashi et al., 2004). Furthermore, in human BeWo cells and first trimester explants, hypoxic induction of GLUT1 and GLUT3 was inhibited by asHIF-1α, and culture in 3% oxygen was shown to increase transepithelial glucose transport (Baumann et al., 2007). Paradoxically, expression of placental GLUT1 is decreased in pregnancies at high altitude (Zamudio et al., 2006).

The placental barrier is also a site of iron transport and recently hypoxia and HIF have been implicated in this process. Iron in serum is mainly transported by transferrin (Dunn et al., 2007) which interacts with the transferrin receptor (TfR) and this complex is internalized by receptor-mediated endocytosis, enabling iron to enter the cell. It has been previously shown that transferrin is up-regulated in response to hypoxia and this is mediated by HIF in hepatoma cells (Rolfs et al., 1997). Similarly, culture of human Hep3B hepatoma cells increases *TfR* mRNA expression via HIF-1 dependent mechanisms (Tacchini et al., 1999). Surprisingly, expression of TfR is decreased in the placentae of pregnancies at high altitude (Zamudio et al., 2006) and in pregnancies complicated by pre-eclampsia (Khatun et al., 2003). However, there is currently no direct evidence for HIF regulation of tranferrin or TfR in the placenta. There are very conflicting results between *in vitro* and *in vivo* studies regarding the effect of hypoxia on GLUTs and TfR in the placenta, perhaps, in part, because of the chronic exposure to hypoxia in pregnancies at high altitude compared with the short-term *in vitro* experiments. Additionally, other factors involved in the regulation of these transporters *in vivo* may be missing from the *in vitro* system, which may warrant further investigation.

The future of placental HIF research

While oxygen regulation of placental development and function and the role of HIFs in these processes is an area of keen interest, the

currently available data are highly confusing. *In vitro* studies examining the effects of oxygen are all diverse in methodology in terms of media composition, cell line versus primary trophoblasts, first trimester versus term placental samples, presence or absence of ECM substrate and timing of exposure. Most studies use 20% oxygen, which is hyperoxic, as a control or comparison group and is irrelevant when considering the physiology of the placenta and all but a few tissues for that matter: it is only useful in attempts to compare it with previous observations in the literature. How then can these data be truly interpreted and related to the *in vivo* situation? It is clear that what is needed is a systematic study looking at the effects of low oxygen (1–3%) compared with physiological control (5–10%) using placental villi from different gestational ages. In addition, given that the effects of prolonged versus acute hypoxia in the placenta are yet to be explored, it will be of great interest to examine this in the future.

Secondly, publication of articles that only report HIF mRNA or protein levels will no longer be sufficient. Ideally, studies examining the regulation of HIF should, where possible, include data on mRNA, protein and DNA binding for both HIF-1 and HIF-2. It is only when more data, such as these for placenta, are available that we will be able to draw accurate conclusions about the role of HIFs in placental biology. In addition, the regulation of HIFs is complex, and involves factors influencing stability, degradation and transcriptional activity of the HIFs. Investigation of other components of the HIF regulatory network, such as asHIF-1α, may offer valuable insights into placental HIF regulation in both normal and pathological pregnancies. Finally, HIF can no longer be considered simply as a transcription factor which is regulated by hypoxia. Future studies examining the regulation of HIF by oxygen and other factors are essential if we are to move forward in this field.

Conclusion

The HIFs are able to function throughout all aspects of normal and abnormal placental differentiation, growth and function; during the first trimester (physiologically low oxygen), during mid-late gestation (where there is adequate maternal blood supply and placental oxygenation) and in pathological pregnancies complicated by placental hypoxia/ischemia. The identity of HIFs as simply hypoxia-responsive transcription factors is an oversimplification and very misleading, as they do much more. During normal pregnancy HIFs respond to complex alterations in oxygen, hormones, cytokines and growth factors to precisely regulate placental invasion, differentiation, transport and vascularization. In the ever-changing environment created during pregnancy, the HIFs appear to act as key mediators of placental development and function and thereby are likely to be important contributors to both normal and adverse pregnancy outcomes.

Authors' Role

K.G.P., K.L.K., A.N.S.-P., J.G.T. and C.T.R. were all involved in reviewing the literature and the preparation of the manuscript.

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