Hypoxia induced HIF-1/HIF-2 activity alters trophoblast transcriptional regulation across gestation

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Declaration

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Abstract

Background: Delivery of oxygen to placental and fetal tissues changes across gestation with a significant increase once maternal blood flow into the placenta is established in late first trimester. Prior to 10 weeks of gestation, fetal and placental development takes place in a physiologically hypoxic environment of 1-2% oxygen tension. Subsequently, the oxygen concentration rises to a level considered normoxic (5-8%) for other tissues following dislocation of extravillous trophoblast cells that have invaded into and initially occluded the maternal spiral arterioles where they remodel the maternal decidual vasculature. Hypoxia inducible factors (HIFs) are transcription factors which respond to changes in oxygen tension and are active in a low oxygen environment. Therefore, oxygen, hypoxia and HIFs play a crucial role in placentation and inadequate trophoblast invasion which when impaired has been associated with pregnancy pathologies such as preeclampsia (PE). The aims of this study were to examine (1) the gene expression profile of first trimester trophoblast cells cultured in different oxygen concentrations and (2) the differential expression of hypoxia responsive genes in the placenta of early and late first trimester, term uncomplicated and term complicated pregnancy.

Methods: HTR8/SVneo trophoblast cells were cultured in 1% (hypoxia), 5% (normoxia) and 20% oxygen (standard culture conditions) for 6 hours. Localisation of HIF-1α and HIF-2α protein in these cells was performed using immunofluorescence. Microarray analyses were undertaken to determine the differential gene expression profile of HTR8/SVneo cells in response to oxygen. Differential expression of selected genes (*IGFBP3*, *IGFBP5*, *MMP1*, *VEGFA*, *P4HA1, P4HA2* and *ANGPTL4*) was validated by qPCR in independent samples. HIF-1α and HIF-2α protein were localised by immunohistochemistry in early (6-8 weeks of gestation) and late (10-12 weeks of gestation) first trimester, and term, placenta tissue samples. Expression of selected genes in placenta samples of early (n=11) and late (n=7) first trimester, term uncomplicated (n=10), term complicated by gestational hypertension (n=8) and term complicated by PE (n=12) pregnancy was also quantified by qPCR.

Results: 290 genes were differentially expressed in HTR8/SVneo cells treated with 1% compared to 5% oxygen. HIF-1α was identified as the top upstream regulator of 41 genes using Ingenuity Pathway Analysis. qPCR validation showed that the expression of *IGFBP3* (P<0.0001), *VEGFA* (P<0.0001), *P4HA1* (P=0.0023), *P4HA2* (P=0.0009) and *ANGPTL4* (P=0.0001) are significantly increased in 1% oxygen compared to 5% oxygen. These genes were predicted to contain HIF-1 transcription factor binding sites. Immunofluorescence showed that HIF-1α is highly expressed in the nucleus of 1% oxygen treated trophoblasts compared to 5% oxygen whereas expression of HIF-2α remained unchanged by oxygen treatment. HIF-1α and HIF-2α were localised to the nucleus and cytoplasm of syncytiotrophoblast, cytotrophoblast and villous stroma of early and late first trimester placenta with an increased expression in the nucleus in early first trimester. Subtle but significantly lower expression of HIF-1α (-4% P<0.013) and higher expression of HIF-2α (+57% P<0.001) was observed in late first trimester placenta compared to early first trimester placenta. qPCR showed that the expression of *IGFBP3* (P<0.0001), *IGFBP5* (P<0.0001), *VEGFA* (P=0.0195) and *ANGPTL4* (P<0.0001) was significantly increased in term control compared to first trimester placenta. No difference was observed in the expression of any genes between term control vs complicated pregnancy.

Conclusion: HIF translocation to the nucleus was confirmed in trophoblasts under low oxygen tension. Genome wide assessment of trophoblast cells *in vitro* identified a large number of differentially expressed genes in different oxygen atmospheres. Of the seven selected genes of interest, *IGFBP3*, *VEGFA* and *ANGPTL4* are known to be involved in trophoblast function and *P4HA1* and *P4HA2* have known roles in cellular proliferation and migration. Together our findings provide additional molecular evidence that expression of HIFs and hypoxia responsive genes is altered with the change of oxygen tension which may mediate known altered trophoblast activity in low oxygen tension.

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Abbreviations

ANGPTL4	Angiopoietin-like 4
ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon receptor nuclear translocator
bHLH	Basic Helix-Loop-Helix
BSA	Bovine serum albumin
cDNA	Complimentary deoxyribonucleic acid
CITED2	p300/CBP interacting transactivator with ED-rich tail 2
CTB	Cytotrophoblast
Cq	Quantification cycle
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
FCS	Fetal calf serum
EVT	Invasive extravillous cytotrophoblast
FIH-1	Factor inhibiting HIF-1
GH	Gestational hypertension
HBSS	Hank's Balanced Salt Solution
HCC	Hepatocellular carcinoma
HIF	Hypoxia inducible factor
HI-FCS	Heat inactivated fetal calf serum
HRE	Hypoxia response element
IGFBP3	Insulin-like growth factor binding protein 3
IGFBP5	Insulin-like growth factor binding protein 5
IL-1β	Interleukin-1β
IPA	Ingenuity Pathway Analysis
IUGR	Intrauterine growth restriction
IVS	Intervillous space
MIOD	Mean integrated optical density
MMP1	Matrix metalloproteinase 1

MOD	Mean optical density
MPP3	Membrane palmitoylated protein 3
MQ H_2O	Milli-Q H_2O
PDGF	Platelet derived growth factor
PHD	Prolyl 4-hydroxylase
P4HA1	Prolyl 4-hydroxylase, alpha polypeptide I
P4HA2	Prolyl 4-hydroxylase, alpha polypeptide II
PE	Preeclampsia
PBS	Phosphate buffered saline
P^{53}	Tumor protein p^{53}
PTB	Preterm birth
qPCR	Quantitative polymerase chain reaction
RAS	Renin-angiotensin system
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
sFlt-1	Soluble fms-like tyrosine kinase-1
SPARC	Secreted protein acidic and rich in cysteine
STB	Syncytiotrophoblast
SRM	Serum reduced media
TAD	Transactivation domain
TGF-β	Transforming growth factor-β
TNF-α	Tumour Necrosis Factor-α
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VEGFA	Vascular endothelial growth factor A
pVHL	Von Hippel–Lindau tumour suppressor gene product

Conference presentations and abstracts arising from this thesis

- **Sultana M Khoda**, Tina Bianco-Miotto, Amanda R Highet, Claire T Roberts. Oxygen and hypoxia-inducible factors: key players in the regulation of trophoblast activity. Annual meeting of Australian Society for Medical Research (ASMR), June 2014, Adelaide, SA, Australia.

- **Sultana M Khoda**, Amanda R Highet, Sam Buckberry, Tina Bianco-Miotto, Claire T Roberts. Effects of oxygen on the expression of hypoxia-inducible factors (HIFs) in the first trimester placenta. 27th Fetal and Neonatal Workshop of Australia and New Zealand (FNWANZ), April 2013, Barossa valley, SA, Australia.

Chapter 1: Literature Review

1.1 Introduction

The placenta is a highly specialised organ unique to pregnancy that supports growth and development of the fetus [1]. Perturbations in placental development can dramatically affect the organ's function and lead to complications in pregnancy. These complications include miscarriage, preeclampsia (PE), intrauterine growth restriction (IUGR) and preterm birth (PTB). The molecular mechanisms underpinning these pregnancy complications are not clearly known but they have been associated with impaired placental development early in gestation. It has become apparent that early placentation is regulated by the combination of oxygen concentration, numerous growth factors, cytokines and angiogenic molecules. While many of these molecules have been proposed to play a central role in placental development, the complex interplay between them and the cross-talk between their signalling pathways remain elusive [2].

1.2 Placenta

The key functions of the placenta are transport, metabolism, protection of the fetus and endocrine action. The placenta transports oxygen, water, carbohydrates, amino acids, lipids, vitamins, minerals and a number of other nutrients to the fetus. It also removes carbon dioxide and other waste products from the fetal circulation to the maternal circulation. It metabolises various substances and releases metabolites into the maternal and/or fetal circulation. The fetus is protected against xenobiotic molecules, infections and maternal diseases through selective transport and passive immunity. Hormones produced in the placenta are secreted into both maternal and fetal circulations to maintain pregnancy and regulate metabolism, fetal growth and parturition [1].

1.2.1 Structure of the mature placenta

The mature placenta is composed of both fetal and maternal tissues. Fetal tissue of the utero-placental unit is derived from the chorionic sac and hence the fetal region is called the chorionic plate. This region contains fetal chorionic blood vessels that branch from the umbilical vessels. Maternal tissue is derived from the endometrium and consequently the maternal region is called the basal plate, carrying transformed maternal blood vessels. The space between the chorionic and basal plates is called the villous region which is composed of highly branched and packed chorionic villi immersed in the intervillous space (IVS) which is perfused by maternal blood from the uterine spiral arterioles. The villi, carrying fetal capillaries, are the main functional unit of the placenta. The majority of maternal-fetal exchange occurs at the terminal regions of chorionic villi (Figure 1.1). The surfaces of the villi are completely covered by a multinucleated syncytium called the syncytiotrophoblast [1, 3]. The syncytiotrophoblast layer is formed by the fusion of villous cytotrophoblasts that lie immediately beneath it

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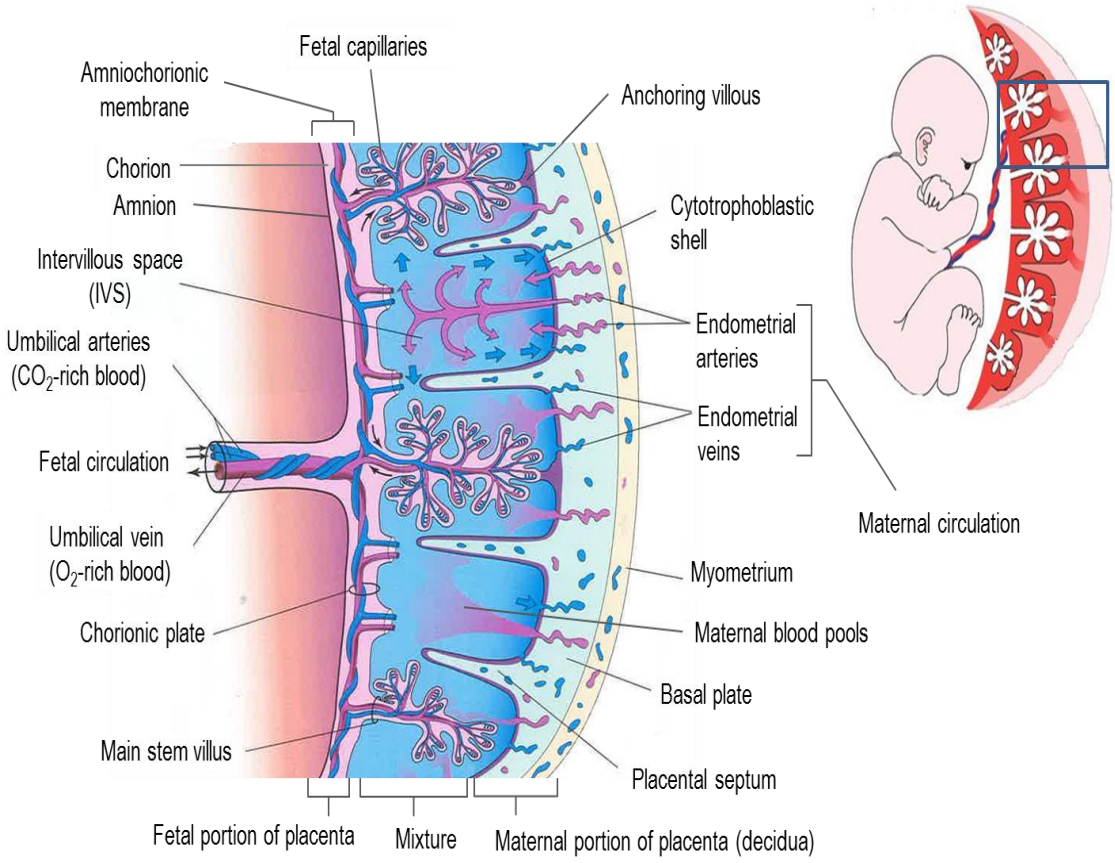

Figure 1.1: Fetal and maternal circulation in the placenta
The cytotrophoblastic shell forms the interface between maternal and fetal tissues. Maternal blood flows into the IVS from endometrial arteries. Exchange of nutrients, gas and wastes takes place between the IVS and fetal blood with the help of different transporter systems located on the syncytiotrophoblast. (Adapted from [4, 5]).

1.3 Pregnancy complications

In Australia, the major pregnancy complications comprise preeclampsia (PE, 8%), intrauterine growth restriction (IUGR, 6%) and preterm birth (PTB, 8%) [6]. PE, IUGR and PTB occur in 19% of first pregnancies (they may occur together or separately within the same pregnancy). In about 6% of all pregnancies, these complications may threaten the lives of the mother and/or her baby [7-9]. These complications compromise both fetal and maternal health. In the case of preeclampsia, the mother suffers from hypertension and proteinuria, while the fetus may be growth restricted. An IUGR baby is at an increased risk of developing a number of diseases

including respiratory disorders, cardiovascular disorders and diabetes later in life [10, 11]. A very preterm baby born less than 34 weeks gestation has a high risk of mortality and morbidity [12].

A large body of literature suggests that pregnancy complications like PE, IUGR and PTB are due to placental insufficiency [13]. Impaired placental development and function impact negatively on the fetus [1, 14-18]. A very recent report showed that placental pathology is a leading cause of infant, neonatal and post neonatal death in the United States [19]. In addition, epidemiologic studies suggest that many cardiovascular, metabolic and endocrine diseases in adult life have fetal origins [20, 21]. Babies delivered from pregnancies complicated by PE and IUGR are more likely to develop heart disease, hypertension and type II diabetes in later life. Women who have had PE also have a high risk for later heart disease. As these pregnancy complications are essentially associated with placental insufficiency, the placenta plays a significant role in programming the fetus for adult diseases [20].

1.3.1 Preeclampsia (PE)

Preeclampsia, a pathology specific to humans, affects approximately 8% of pregnancies and hence is a major contributor to both maternal and fetal morbidity and mortality [22, 23]. It occurs after 20 weeks of gestation and is characterized by maternal hypertension (blood pressure ≥ 140/90 mmHg), proteinuria (≥300mg/day), variably multi-organ complications and fetal growth restriction [22, 24]. It is suggested that the placenta plays a significant role in the development of PE as its only cure is the delivery of the placenta. Clinically there are two types of PE- early-onset and late-onset PE, resulting from heterogeneous causes [25]. Early-onset PE develops before 34 weeks of gestation and has a stronger association with intrauterine growth restriction and iatrogenic preterm birth [26]. Early-onset PE is suggested to be strongly related with placental dysfunction whereas late-onset PE, developing after 34 weeks of gestation, has a weaker association with placental dysfunction and is considered a maternal

disease only [27, 28]. In the first trimester of pregnancy, PE is initiated with impaired invasion of trophoblast into the decidua and remodelling of the spiral arteries leading to inadequate placentation and hypoxia in the placenta [29]. It is highly suggested that hypoxia and factors associated with hypoxia play roles in development of PE [30, 31].

1.3.2 Gestational hypertension (GH)

Gestational hypertension or pregnancy-induced hypertension (PIH) is defined as the hypertension (blood pressure ≥ 140/90 mmHg) that developed after 20 weeks of gestation without the presence of proteinuria. Previously, GH was considered as less important indicator for adverse pregnancy outcomes due to the absence of proteinuria [32]. However, severe gestational hypertension has been reported to be highly associated with lower gestational age at delivery and low-birth-weight infants when compared to mild GH or mild PE [33]. A study conducted in women with GH and PE showed that increased rate of preterm and small-for-gestational-age infants was delivered from women with severe hypertension and the presence of proteinuria did not influence the perinatal outcome [34].

1.3.3 Intrauterine growth restriction (IUGR)

Intrauterine growth restriction is defined by fetal birth weight of less than 2500g and comprises 6% of pregnancies. It is a global problem and is one of the most serious perinatal syndromes. Intrauterine growth restricted fetuses and neonates are at higher risk for perinatal mortality and morbidity [35]. The placenta in pregnancies complicated by IUGR is characterized by poor villous growth and development with impaired vasculogenesis [36], and a small surface area of syncytiotrophoblast that results in insufficient transport of nutrients and gases across the placenta to the fetus [23, 37]. IUGR also occurs in approximately 30% of preeclampsia cases [23]. IUGR has been associated with permanent risk for a range of childhood and adult disorders including cognitive impairment [38], hypertension, obesity, type II diabetes, cardiovascular and renal diseases [10, 11, 39, 40].

1.3.4 Preterm birth (PTB)

Preterm birth, designated as delivery prior to 37 weeks of gestation, is a worldwide health issue. Every year, 15 million babies are delivered preterm and more than 1 million children die because of complications caused by PTB. The rates of PTB are increasing in almost all countries [41]. Those who survive are at higher risk of developing hearing, learning and visual disability and diseases like hypertension and diabetes in later life [42]. PTB is clinically classified as (1) spontaneous PTB (spontaneous onset of preterm labour or premature rupture of the membranes) and (2) indicated PTB (induced or elective caesarean birth before 37 weeks of gestation for maternal or fetal reasons) [43, 44]. In about 70% cases, PTB occurs spontaneously. The common causes of PTB are multifactorial and include maternal, placental or fetal complications such as infections, diabetes, high blood pressure and premature rupture of the membranes [41]. A number of studies have also indicated genetic, epigenetic and environmental risk factors contribute to PTB [43, 45, 46]. Fetal sex also has an effect with male fetuses having an increased risk of being delivered by spontaneous PTB [47, 48]. However, in half of the cases, the causes for spontaneous PTB remain unknown [49]. Evidence is growing to suggest that the processes of implantation and placentation in early pregnancy play crucial roles in the pathophysiology of PTB and premature rupture of the membranes [13, 50, 51].

1.4 Trophoblast

After fertilization in the ampulla of the fallopian tube, the human embryo differentiates into the blastocyst within 4 to 5 days at which time it enters the uterus. The blastocyst consists of trophoblast cells which form the outer layer, blastocoel and inner cell mass. In the uterus, the blastocyst becomes attached to the uterine luminal epithelium followed by penetration of the epithelium and finally becomes embedded in the maternal stroma, (called the decidua during pregnancy). Implantation occurs on day 5 to 6 at which time the trophoblasts differentiate into two layers: an inner cytotrophoblast (CTB) layer and an outer multinucleated

syncytiotrophoblast (STB) layer which is formed by the fusion of CTBs. Following complete penetration of the embryo on day 10, early chorionic villous formation begins within 15 to 16 days [4]. Depending on the morphology, location and subsequent function during human placentation, trophoblast cells are classified as: (a) villous CTB - the inner layer (b) villous STB - the outer layer (c) anchoring CTB – found at the tips of anchoring villi and (d) invasive extravillous CTB (EVT) – found in the implantation site (Figure 1.2). Within the villi of the human placenta, there always exists a population of undifferentiated CTBs available for differentiation when necessary at all gestational ages [52, 53]. In anchoring villi, CTBs form the cell column through proliferation, and the EVTs detach from these columns and migrate into the decidua forming interstitial CTB. Another population of migratory trophoblast is endovascular CTB which migrate into spiral arterioles within the decidua and play an essential role in vascular remodelling [2, 54].

1.4.1 Trophoblast invasion

Placental development is a complex process involving the regulation of trophoblast proliferation, differentiation and invasion. These trophoblast functions enable a sufficient blood supply and large surface area for exchange for the developing placenta and the growing fetus. Although oxygen is essential for late gestation placental function and fetal growth, paradoxically, in early gestation, the embryo and placenta differentiate in a relatively low oxygen environment. Initially, endovascular CTBs invade and plug the uterine spiral arterioles. This results in limited or no maternal blood flow to the intervillous space (IVS). Endovascular CTBs occlude the spiral arterioles but late in first trimester (approximately 10 to 12 weeks of gestation), the plugs are displaced permitting maternal blood to flow to the IVS (Figure 1.2) [55]. The maternal endothelium and smooth muscle are remodelled and the vessels become dilated, compliant and lose vasoreactivity. This results in a 12-fold increase in uterine blood

flow compared to the non-pregnant state to fulfil the demands of the growing fetus in late gestation [56].

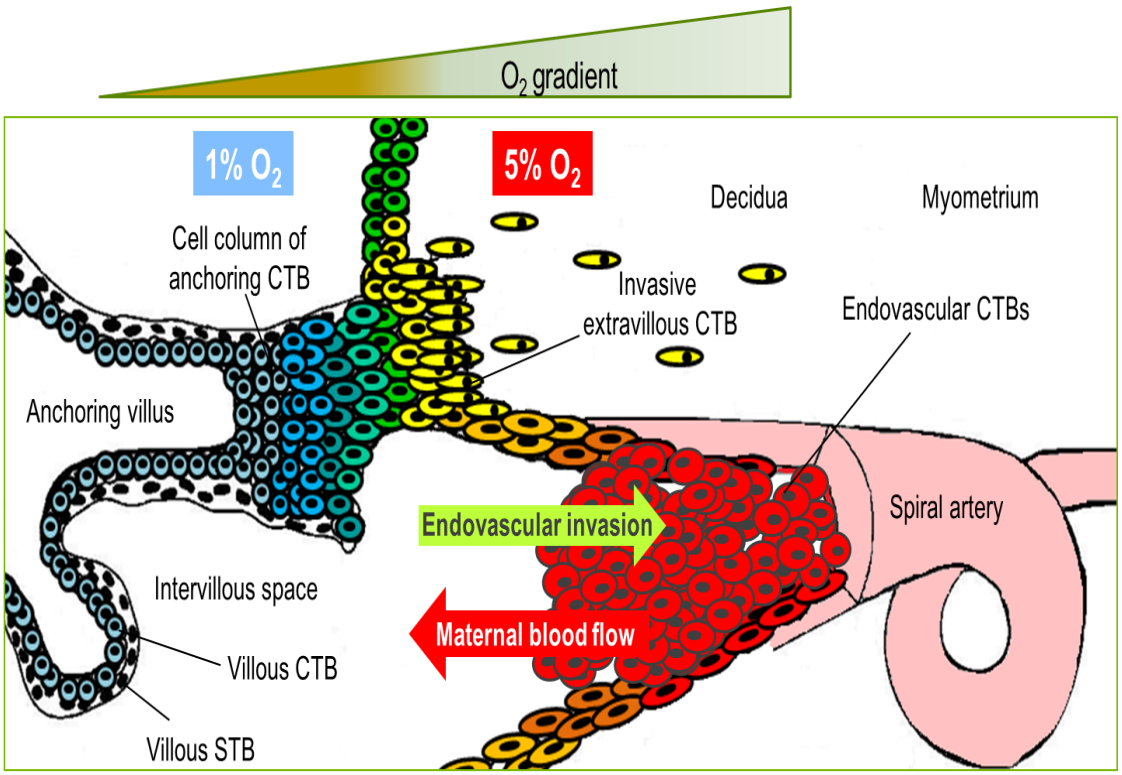

Figure 1.2: Placental development
The endovascular CTBs replace the endothelium of maternal spiral arteries, smooth muscle cells are lost resulting in dilated compliant vessels which allow maternal blood to flow into the IVS (created from [54] and Highet, unpublished).

1.4.2 Factors affecting trophoblast invasion

Both trophoblast-derived and maternal factors control trophoblast invasion in a spatio-temporal manner [57, 58]. A number of factors have been implicated in the regulation of trophoblast invasion. These include oxygen, hypoxia inducible factors (HIFs) [59], a variety of growth factors [2], members of the renin-angiotensin system (RAS) [60], cytokines, cell adhesion molecules (integrins, cadherins), extracellular matrix (ECM) components and their regulators including collagen, vitronectin, matrix metalloproteinases (MMPs), urokinase plasminogen activator (uPA), plasminogen and hormones [61-63]. Any impaired regulation of trophoblast functions mediated by any of these factors may result in adverse pregnancy outcomes.

1.4.3 Influence of oxygen on trophoblast invasion

After the onset of maternal blood flow, oxygen tension is increased within the IVS from 18 mmHg (2.5%, at 8 weeks) to approximately 60 mmHg (8.5%, at 12 weeks) [64-66], leading to a burst of oxidative stress in the placenta, particularly in the STB [64]. This well-oxygenated environment plays a physiological role in stimulating trophoblast differentiation. However, early onset of maternal blood flow into the IVS results in premature increased placental oxidative stress [67, 68] and is associated with early pregnancy loss [67-70]. This premature oxidative stress is caused by low levels of antioxidant enzymes in the placenta at 8–10 weeks of gestation [71, 72]. During this time a low oxygen environment is essential for adequate trophoblast invasion and plugging of the uterine arterioles. Insufficient trophoblast invasion and defective vascular remodelling of spiral arteries in the first trimester can lead to impaired placental perfusion, and chronic ischemia and hypoxia later in gestation [73]. These detrimental effects may lead to development of PE, IUGR, and PTB [74, 75]. Therefore, effects of oxygen concentration are critical in placental and fetal development, and pregnancy complications.

1.5 Hypoxia and hypoxia inducible factors (HIFs)

Almost every mammalian cell type has the capacity to survive in a low oxygen environment [76] and during the very early stages, the mammalian embryo develops in a hypoxic environment [77] . The transcription factors which are activated in low oxygen conditions are called Hypoxia Inducible Factors (HIFs). HIFs are a family of basic Helix-Loop-Helix (bHLH) transcription factors. HIFs occur as a heterodimer consisting of two subunits, HIF-α and the aryl hydrocarbon receptor nuclear translocator (ARNT; also known as HIF-1β) [78-80]. HIFs interact through two PER-ARNT-SIM (PAS) domains, bind DNA via N-terminal basic bHLH domains and activate transcription through C-terminal transcriptional transactivation domains (TADs) [77]. Three HIF-α genes (*HIF1A*, *HIF2A* and *HIF3A*) have been identified in mammals [77]. *HIF1A* is expressed ubiquitously while its paralogs *HIF2A* and *HIF3A* are expressed in a more

restricted pattern [81] suggesting that HIF-1α plays a role in homeostasis whereas HIF-2α and HIF-3α may be involved in more specialized and tissue specific regulatory functions [82].

1.5.1 HIFs in the placenta

Hypoxia and HIFs have been implicated as the key regulators in placental development and function [59, 83, 84]. In normal pregnancy, early fetal and placental development takes place in a hypoxic environment. However, late gestation hypoxia is associated with pregnancy complications such as PE and IUGR [85]. Therefore, appropriate regulation of HIFs in the placenta is critical in both normal and complicated pregnancies. HIFs regulate placental vascularisation, trophoblast invasion and differentiation through responding to the change in oxygen supply in the placenta [86, 87]. Interestingly, HIFs also respond to non-hypoxic stimuli such as hormones, growth factors, renin-angiotensin system and cytokines which are important regulators of placental development [59, 88]. *HIF1A* mRNA and protein are expressed throughout pregnancy with the highest level of *HIF1A* mRNA during 7-10 weeks of gestation while the expression of *HIF2A* mRNA and protein are stable throughout gestation [89]. Moreover, HIFs are regulated differently in different hypoxic conditions [59]. It is likely that the expression and regulation of HIFs are highly complex, and the interactions among HIFs and other regulatory pathways need to be elucidated to better understand placental pathology.

1.5.2 Regulation of HIFs during hypoxia and normoxia

Low oxygen tension, regarded as hypoxia, induces nuclear translocation and dimerization of HIF-1α and HIF-2α with ARNT forming HIF-1 and HIF-2 followed by binding to the hypoxia response element (HRE) of target genes (Figure 1.3 A) [59, 77, 88]. This HRE has the core consensus sequence 5'-CGTG-3' in responsive genes [90]. The expression of a large variety of genes which are involved in erythropoiesis, angiogenesis, cell proliferation, apoptosis and glucose metabolism is mediated by HIFs [78, 91].

ARNT is constitutively expressed in mammals, but the expression and activity of HIF-1α depends on cellular oxygen concentration [77]. Under normoxic conditions (tissue oxygen concentration > 5%), HIF-1α has a half-life of less than 5 min [79]. Normoxia induces the hydroxylation of HIF-1α at conserved proline residues (HIF-1α Pro564 and Pro402) by prolyl-4-hydroxylases (PHD1-3), located within a unique oxygen-dependent degradation domain. The von Hippel–Lindau (VHL) tumour suppressor gene product (pVHL) then binds HIF-1α [92-94]. pVHL functions as the recognition component of an E3 ubiquitin protein ligase complex leading to polyubiquitination and proteasomal degradation of HIF-1α (Figure 1.3 B) [95-97].

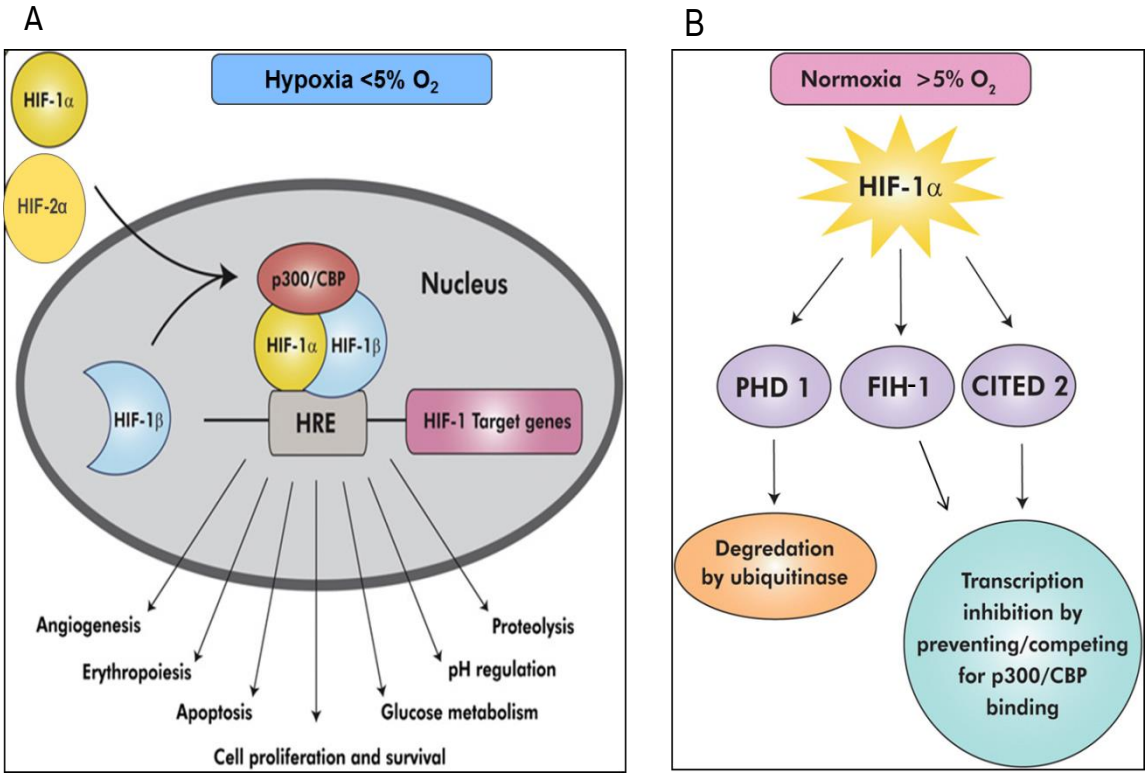

Figure 1.3: HIF regulation under hypoxia (A) and normoxia (B)
Upon nuclear translocation, HIF-1α dimerizes with HIF-1β (ARNT) forming a protein complex, which binds to the associated HRE of HIF-1 target genes leading to transcription. B) HIF regulation under normoxia. Proteosomal degradation of HIF-1α by three pathways (1) PHD1 via hydroxylation and ubiquitination, (2) FIH-1 and (3) CITED2, through preventing or competing with HIF-1α for binding with p300/CBP [59, 88]).

Additionally, HIF-1α has two oxygen-dependent inhibitors, factor inhibiting HIF-1 (FIH-1) and p300/CBP interacting transactivator with ED-rich tail 2 (CITED2) [77]. FIH-1 is an Fe(II)-dependent asparaginyl hydroxylase. During normoxia, it hydroxylates a conserved asparagine residue in the TAD region and inhibits the interaction of HIF-1α and HIF-2α with transcriptional coactivators p300/CBP Figure 1.3B) [98, 99]. CITED2 functions as a negative regulator of HIF-1α and actively competes with HIF-1α for p300/CBP binding (Figure 1.3 B) [100].

During hypoxia, the rate of prolyl and asparaginyl hydroxylation is reduced leading to HIF mediated gene transcription [77]. In hypoxia, CITED2 is activated through HRE [100] suggesting a possible negative feedback to HIF-1α transcriptional activity [59]. CITED2 may also play a vital role in regulating HIF function during placental development and differentiation, as impaired trophoblast invasion and inadequate placental blood supply were shown in CITED2 null mice [101].

Furthermore, HIFs respond differently in different hypoxic conditions [59]. Under acute hypoxia, HIF-1α and HIF-2α become stable inducing the transcription of their target genes. In prolonged hypoxia, HIF-1α and HIF-2α stimulate the expression of the antisense transcript of HIF-1α (asHIF-1α). This asHIF-1α destabilizes *HIF1A* mRNA resulting in low levels of HIF-1α mediated gene transcription and HIF-1α protein [102]. Conversely, *HIF2A* mRNA lacks AU rich elements, and is not destabilized by asHIF-1α. Consequently, HIF-2α mediated transcription and HIF-2α protein are more available under prolonged hypoxia [103].

1.5.3 HIFs in trophoblast invasion

During the first trimester, hypoxia and HIFs are essential for trophoblast functions including proliferation, invasion and differentiation [77, 104]. This hypoxic environment regulates invasion through influencing the proliferation and apoptosis of trophoblasts [105]. The human placenta expresses HIF-1α and HIF-2α in the STB, villous CTB and fetoplacental vascular endothelium

[90]. Several experiments have reported HIFs as essential players in the regulation of trophoblast invasion [86, 104]. Silencing of *HIF1A* mRNA caused significantly reduced invasion and migration in JEG-3 human choriocarcinoma cell line, which has several trophoblast-like characteristics [104]. *Hif1α* -/- *Hif2α* -/- mice showed 17% decreased trophoblast invasion in placenta compared to wild type [86]. The hypoxia/HIF signalling pathway has been identified in controlling differentiation and invasion of the trophoblast stem cells of rat (reviewed in [106]). Severe placental defects including shallow placental invasion and altered trophoblast tissue formation leading to death after day E10.5 were observed in *Arnt*-/- [107] and *Hif 1a*-/-*Hif 2a*-/- mouse embryos [86, 108]. Expression of pVHL and HIF-2α, but not HIF-1α, were upregulated by hypoxia *in vitro* but decreased in a presumably well-oxygenated environment *in situ* in human CTB during invasion of the maternal decidua [54]. Although differential regulation of HIF-1α and HIF-2α in trophoblasts has been widely studied in animal models, disparity in the location of trophoblasts during different stages of human pregnancy is yet to be shown.

A number of factors expressed by trophoblasts and maternal decidua, which are involved in the regulation of trophoblast invasion and migration, are also HIF target genes [59]. Insulin-like growth factor-2 (IGF2) is one of the important factors regulating EVT migration [109] and it is regulated by HIF among other things [110]. Though there is no direct evidence for regulation of IGF2 by HIF in the placenta, *in vivo* hypoxia at term is associated with increased *IGF2* expression [111]. Additionally *Hif1α* and *Hif2α* have been shown to be differentially regulated by oxygen and *IGF2* in murine trophoblasts [112]. Transforming growth factor-β (TGF-β) isoforms are also major factors regulating trophoblast functions [113]. Expression of TGF-β3 is induced by hypoxia both *in vivo* and *in vitro* and is regulated by HIF-1α [114, 115]. Interleukin-1β (IL-1β) and Tumour Necrosis Factor-α (TNF-α) increased HIF-1α-mediated vascular endothelial growth factor (VEGF) secretion in normal human trophoblast cells [116]. MMPs, uPA, the uPA receptor (uPAR) and uPA inhibitors are another group of factors that play a key

role in trophoblast migration, invasion and remodelling of spiral arteries [117, 118]. Exposure of HTR-8/SVneo trophoblasts to less than 1% oxygen *in vitro* results in increased uPAR mRNA and protein expression, and enhanced trophoblast invasion [119]. Conversely, a recent study showed that exposure of villous explants to 3% oxygen decreases uPAR activity and inhibits EVT invasion [120]. It was suggested that these conflicting results were due to different cell types and the methodology [120]. However, further evidence is essential to determine the combined regulation of HIFs and other factors in different oxygen environment during trophoblast invasion.

1.5.4 HIFs in pregnancy complications

Though hypoxia is essential for trophoblast invasion early in pregnancy, hypoxia later in gestation resulting from inadequate maternal blood supply to the placenta is associated with complications such as PE and IUGR [31, 121, 122]. This inadequate blood supply is the result of inadequate trophoblast invasion and remodeling of the spiral arteries. Hypoxia leads to the up-regulation of HIFs and their target genes [31]. A large body of literature showed altered expression of HIFs and their target genes in the placentas of complicated human pregnancies. HIF-1α and HIF-2α are up-regulated [123, 124] while oxygen-dependent proteosomal degradation of HIF-1α and -2α is impaired in the villous placenta of preeclamptic pregnancies compared to normal term pregnancies [31], and may be due to proteosome dysfunction [125]. HIF-1α regulates TGF-β3, a target gene induced by hypoxia in trophoblast cells [88, 114]. Both HIF-1α and TGF-β3 are up-regulated in PE and IUGR [126-128]. p53 is a central tumor suppressor gene which activates the transcription of a wide variety of genes under hypoxia [129]. Placentas of pregnancies complicated by fetal growth restriction (FGR) showed increased apoptosis and over-expression of p53 [130]. Similarly, VEGF, another HIF target gene is over-expressed in the placenta of pregnancies complicated by PE and IUGR [126].

These data suggest that interacting regulations of HIFs and their target genes mediate the pathophysiology of the placenta.

1.6 Studies on different oxygen tensions and trophoblast invasion

Investigating the effect of different oxygen tensions in trophoblast invasion has been difficult as this process is also regulated by various factors such as autocrine and paracrine growth factors, cytokines and surrounding environmental factors. In addition, studying the regulatory effects of these factors including oxygen in trophoblast invasion *in vitro* is also hard as it is difficult to get a pure population of EVT in primary culture due to technical difficulties [131]. Genbacev et al. 1996 showed that lowering oxygen tension to 2%, compared to 20%, causes the failure of early gestation CTB stem cells to invade an extracellular matrix substrate which mimics the alterations in CTB invasion thought to occur in PE [132]. A study conducted in 4 different trophoblast cell lines (HTR8/SVneo, SGHPL4, JEG3 and JAR) showed that invasion of HTR8/SVneo and JEG3 cells was increased after 24 hours but was inhibited by 72 hours when cultured in 3% oxygen compared to 20% oxygen [131]. Whereas invasiveness of SGHPL4 cells was inhibited after 24 hours when cultured in 3% oxygen compared to 20% and invasion of JAR cells remain unchanged in different oxygen atmospheres indicating differential responses of different cell lines to oxygen tension[131]. Hence, it is essential to further investigate the regulatory effect of different oxygen tensions on trophoblast invasion both *in vivo* and *in vitro*.

1.7 Interactions between oxygen, HIF1A and various factors

In the last two decades many studies have shown the individual regulatory effects of different factors on trophoblast activity. Some of these factors promote trophoblast invasion [57, 58, 133, 134] while others reduce invasion [135, 136] or cause both effects. In addition, regulatory binding proteins like insulin-like growth factor binding proteins (IGFBPs) [62], soluble fms-like tyrosine kinase-1 (sFlt-1) [137], secreted TGF-β co-receptor have been identified in the regulatory network of trophoblast invasion. Moreover, HIF-1α stabilisation is altered in

response to oxygen concentration, components of the Renin-angiotensin system (RAS), growth factors and cytokines all of which regulate trophoblast function [88, 112, 138-140]. However, there are limited studies showing the interactions of these factors in trophoblast activity. The effect of different oxygen environments on these interactions also remains elusive and could explain some conflicting data. In addition, it is unknown whether there is a master regulator of all these factors. Preliminary analysis of existing data using Ingenuity Pathway Analysis (IPA) suggests increased *HIF1A* expression is important for many of the regulators of placental development discussed so far (Figure 1.4). The analysis was done without specifying any species or tissue. However, we propose that HIF-1α is the master regulator of trophoblast invasion.

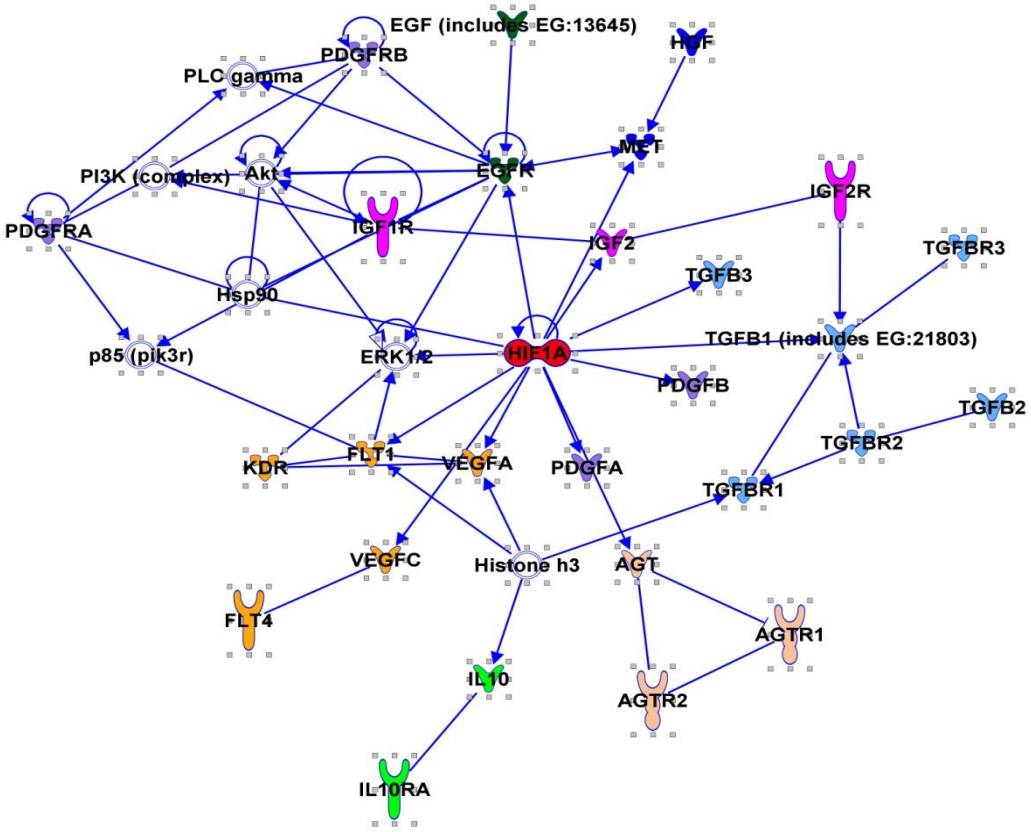

Figure 1.4: Known interactions between HIF1A and other factors
HIF1A and other factors were placed into IPA to investigate direct relationships between these factors. This figure indicates that HIF1A has direct effects on many other factors associated with trophoblast invasion. The different colours indicate different family of genes.

1.8 Conclusion

Early placental development determines the fate of the fetus during gestation, as well as later in life. Currently oxygen and hypoxia are believed to play the most vital roles in fetal and placental development. However, the interactions between varied oxygen tensions and different factors responsive to the change of oxygen tensions are still unclear. This study will investigate trophoblast gene expression responses to different oxygen tensions *in vitro* and localise differentially expressed genes *in vivo* across gestation and in placenta from normal pregnancies and those with complications. These interactions are important for healthy fetal development, as well as in pregnancy pathologies such as PE and IUGR. This research will help to improve our understanding about the placental origin of pregnancy complications related to life-threatening diseases in adult life.

Chapter 2: Gene expression profiling in HTR8/SVneo trophoblast cells in response to different oxygen concentrations

2.1 Introduction

Trophoblasts are specialised cells which originate from the outer layer of the blastocyst that mediate implantation and placentation through different functions. One of these functions is the invasion and remodelling of spiral arteries of maternal decidua at the end of first trimester of pregnancy. Remodelling of maternal spiral arteries is essential for sufficient blood and oxygen flow to the intervillous space of the placenta. In the early stages of first trimester, invasive extravillous trophoblasts (EVT) invade the maternal spiral arteries and form plugs that occlude the vessels creating a low oxygen environment within the intervillous space (IVS). At this stage oxygen tension is approximately 18 mmHg (2.5%) [64, 65]. This low oxygen environment is necessary to protect the embryo from oxidative stress as the placenta contains very low levels of antioxidants during this period [72]. As pregnancy progresses, the plugs start to be displaced allowing maternal blood to flow to the IVS which results in a rise of oxygen tension. Blood flow begins to be established at approximately 10 weeks of gestation and by 12 weeks the oxygen tension is approximately 60 mmHg (8.5%) [64, 65]. Although the physiologically low oxygen environment, which is often called hypoxia, is vital for early placentation while hypoxia at later stages is associated with adverse pregnancy outcomes like preeclampsia (PE) and intrauterine growth restriction (IUGR). Insufficient trophoblast invasion and remodelling of spiral arteries have been shown to be the leading causes of PE and IUGR [85, 141, 142].

Hypoxia inducible factors (HIFs) are a family of basic Helix-Loop-Helix transcription factors which mediate responses to the change in oxygen tension. HIFs are heterodimers consisting of HIF-α and the aryl hydrocarbon receptor nuclear translocator (ARNT; also known as HIF-1β) [78-80]. In mammals, three genes have been identified that encode the HIF-α subunit (*HIF1A*,

HIF2A and *HIF3A*) [77]. As low oxygen is essential for early trophoblast invasion, hypoxia and HIFs play an essential role in placental development. The level of HIF-1α and HIF-2α protein expression changes through gestation as oxygen concentration changes [90]. Expression of *HIF1A* mRNA has been shown to remain constant while the expression of *HIF2A* mRNA was increased as the pregnancy proceeds [90]. Altered regulation of HIFs may result in impaired trophoblast invasion leading to adverse pregnancy outcomes [31, 85, 123]. A number of HIF target genes such as members of the IGF family [110], the VEGF family [143], the uPA system [119] and the TGF family [114] play critical roles in trophoblast functions and placental development [113, 119, 144, 145]. A growing body of literature suggests that HIFs can also be regulated by different factors other than hypoxia such as platelet derived growth factor (PDGF) [146], transforming growth factor-β1 (TGF-β1) [147], epidermal growth factor (EGF), insulin [148], insulin like growth factor-1 and -2 (IGF1 [146] and IGF2 [112]), interleukin- 1β, progesterone and angiotensin-II at the mRNA and/or protein level through feedback pathways (reviewed in [59]). Several studies have shown the importance of HIFs in placental development especially in trophoblast invasion, migration, differentiation and angiogenesis [86, 87, 104, 149]. Knockout of *Hif1α* and *Hif2α* reduces trophoblast invasion and migration in mice [86]. Increased expression of HIF-2α but not HIF-1α or HIF-1β has been shown in preeclamptic placenta compared to normal term placenta [123].

The interaction between HIFs and different factors is critical in healthy placental development, as well as in placental pathology. Though the role of oxygen and the influence of hypoxia are key regulators of trophoblast function, how they regulate other factors is still unclear. Moreover, it is likely that HIF-1α plays a crucial role in trophoblast functioning. However, how HIF-1α regulates other hypoxia responsive factors also needs to be clarified. Here, we will examine differential gene expression patterns in different oxygen environments in HTR8/SVneo trophoblasts, which have been used widely for investigating first trimester cytotrophoblast

behaviour, using microarray and quantitative PCR Microarray analyses reveal global gene expression patterns of HTR8/SVneo cells in response to different oxygen tension. We will also determine the localisation of HIFs and differential protein expression in different oxygen tensions to correlate with gene expression. The chosen oxygen concentrations represent varied oxygen tension within the placenta during first trimester as well as with standard cell culture conditions. Our study will provide further insight into the effect of varied oxygen concentrations within the placenta which is likely regulated by HIFs.

2.2 Materials and Method

2.2.1 Cell culture

HTR8/SVneo cell line was produced from human first trimester trophoblast cells and immortalised with simian virus 40 (SV40) and a kind gift from Professor Charles Graham, Queen's University, Ontario, Canada [150]. The HTR8/SVneo cells were maintained at 37ºC (humidified), 20% oxygen and 5% carbon dioxide in RPMI-1640 media (Sigma-Aldrich, St Louis, USA) with 10% v/v heat inactivated fetal calf serum (HI-FCS) and 2 mM L-glutamine (Sigma-Aldrich, St Louis, USA). When the cells reached 80-90% confluence, the media was discarded and the cells were washed with HBSS (Hank's Balanced Salt Solution) (Life Technologies, Carlsbad, CA, USA) twice. Cells were dislodged from the flask using 1X trypsin-EDTA (Life Technologies, Carlsbad, CA, USA). The action of trypsin was inactivated by RPMI-1640 media supplemented with 10% fetal calf serum (FCS). From these cells, 300,000 HTR8/SVneo per well were cultured in serum reduced media (SRM, containing RPMI-1640 media, 0.5% HI-FBS and 2 mM L-glutamine) in a 6-well culture plate for RNA and protein extraction and in chamber slides for immunofluorescence. Six replicates from each experimental group were run in each experiment. All the 6-well culture plates and chamber slides were incubated at 1%, 5% or 20% oxygen, 5% CO_2 at 37ºC (humidified) for 6 hours. 1%, 5% and 20% oxygen reflect early first trimester (6-8 weeks of gestation), late first trimester (10-

12 weeks of gestation) placental oxygen environment and standard culture conditions respectively [65, 66, 151]. 1% and 5% oxygen conditions were achieved using a sealed modular secondary chamber attached to a controlled gas cylinder containing a mix of 1% or 5% oxygen and 5% CO_2 in nitrogen at a rate of 5L/min for 10 min. To confirm the appropriate oxygen atmosphere inside the chamber, an MX 3001 oxygen sensor (Teledyne Analytical Instruments, Industry, CA, USA) was used. After 6 hours, the media was discarded and the cells were harvested in 1 ml TRIzol reagent (Ambion, Carlsbad, CA, USA) per well for RNA extraction. Six hours of culture was chosen as previous real-time invasion assays using HTR8/SVneo trophoblasts in our laboratory show sufficient invasion during this time (data not shown). For protein extraction, media was discarded from the culture plate and the cells were washed with HBSS. The cells were dislodged from the bottom of the well by adding 1X trypsin-EDTA followed by trypsin inactivation as above. The suspension was centrifuged at 5000 rpm for 5 min, supernatant was discarded and the remaining pellet was used for protein extraction. From the chamber slides, media was discarded and the slides were dried and fixed by methanol. The slides were stored at -80ºC for later immunofluorescence studies.

2.2.2 Immunofluorescence

Immunofluorescence was performed to localise HIF-1α and HIF-2α protein on HTR8/SVneo trophoblast cells treated with different oxygen atmospheres. Chamber slides were taken out from -80°C, air dried and washed in Milli-Q (MQ) H_2O and 1X PBS for 5 min (3 times). The slides were quenched with 6% H_2O_2 for 1 h to remove endogenous peroxidases. The cells were blocked with diluent (1% BSA and 10% pig serum in 1X PBS) for 1 h. Finally, the cells were labelled with HIF-1α (NB100-134, Novus Biologicals, 1:1500) or HIF-2α (NB100-122, Novus Biologicals, 1:1500) primary antibody and diluent as negative control and incubated overnight at room temperature in a humidified chamber. On the following day, the slides were incubated with secondary antibody (Goat anti Rabbit, 1:500, Dako Cytomation, Glostrup,

Denmark) for 1 h. The slides were incubated with Streptavidin-Cy3 (1:100, Sigma-Aldrich, St Louis, USA) in the dark for 1 h. Finally, slides were incubated with DAPI for 5 min. Between steps, the slides were washed by MQ H_2O and 1X PBS for 5 min (3 times). The slides were mounted by NorthernLights Guard Mounting Media (R&D systems, Minneapolis, MN, USA). Images were captured using an Eclipse Ni-U microscope (Nikon, Tokyo, Japan) and NIS-Elements Imaging software v 4.10 (Nikon, Tokyo, Japan). Excitation and emission wavelengths used for imaging DAPI were 358 nm and 461 nm, and those for Cy3 were 550 nm and 570 nm, respectively.

2.2.3 RNA extraction from HTR8/SVneo

RNA was extracted from HTR8/SVneo trophoblast cells using TRIzol reagent (Ambion**,** Carlsbad, CA, USA) and purified using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quality was confirmed by agarose gel electrophoresis and the concentration was determined using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE). RNA integrity was determined using the Experion Automated Electrophoresis Station (Bio-rad, Hercules, CA, USA). The RNA was sent to the Australian Genome Research Facility Ltd (AGRF, St Lucia, QLD, Australia) for microarray analyses (see below). RNA was also extracted from an independent set of samples, cultured in a separate experiment, for qPCR validation using TRIzol reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA integrity was confirmed by agarose gel electrophoresis and the concentration of RNA was determined using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE)**.**

2.2.4 HTR8/SVneo microarray

For microarray analyses, four RNA replicates from each experimental group were sent to the Australian Genome Research Facility (AGRF), University of Queensland, St Lucia, QLD, Australia. Microarray was performed using Illumina TotalPrep™ RNA Amplification Kit (Ambion,

Carlsbad, CA, USA) for labelling and Illumina HT-12 Version 4 Human Bead Gene Expression Arrays (San Diego, CA, USA) for hybridisation. These arrays target more than 47,000 probes which can provide coverage of 28,688 transcripts and are able to detect 1.35 fold changes across more than 3 logs of dynamic range. Scanning was performed by AGRF using the Illumina Sentrix with Genome Studio Version 1.9.0 software.

2.2.5 Analysis of microarray data

The probe-labelled raw data were background-corrected, log2 transformed and variance stabilized using lumiR package of R Bioconductor (www.Bioconductor.org) [152, 153]. Normalised probe intensities were analysed by ANOVA using Partek Genomic Suite™ software, version 6.6 build 6.12.0420 (Partek Inc., St. Louis, MO, USA). The level of significance of varied normalized expression values between sample groups was calculated using ANOVA. Fold change was calculated as mean ratio of gene expression. Probes with an unadjusted p-value ≤ 0.05 and an absolute fold change ≥ 1.5 were defined as differentially expressed. The fold difference of 1.5 was chosen as an arbitrary cut-off above which microarray data are reliable. Gene ontology enrichment analysis on the list of differentially expressed probes was performed in Partek. A functional group with an enrichment score >1 was considered as over expressed and a score > 3 was considered as significant over expression. Differentially expressed gene lists were analysed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com) and Panther Classification System Version 8.1 (Thomas lab, University of Southern California, www.pantherdb.org) [154]. Presence of HIF-1α:ARNT(HIF-1β) transcription factor binding sites on the genes of interest was detected using oPOSSUM (http://opossum.cisreg.ca, Version 3.0).

2.2.6 Reverse transcription, quantitative PCR (qPCR)

RNA (4 µg) was treated with TURBO DNA-free™ DNase treatment kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's protocol to remove DNA. To check for genomic DNA

(gDNA) contamination a PCR was run using Faststart Universal Master Mix (Roche, Mannheim, Germany) and gDNA primers (IGF2R promoter region; F-GCCTCTTCTTGTTAATTTCCCTGTT, R- TTCAGTTTCTCCACAGACATTCAA, of 95 bp amplicon length) [155]. Absence of gDNA was confirmed with the absence of amplification of DNase treated RNA. Cycling conditions were: 95°C for 10 min, then 40 cycles of 98°C for 5 sec and 60°C for 20 sec.

Reverse transcription of DNase treated RNA (500 ng) was performed using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Three RNA replicates from each experimental condition were reverse transcribed. The resultant cDNA was diluted 1:2 with nuclease free water. With this diluted cDNA, qPCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA) and specific TaqMan Gene Expression Assays (Table 2.1) (Applied Biosystems, Carlsbad, CA, USA) to validate the expression of genes of interest selected from microarray data. The reaction volume was 10 µl containing 2 µl of cDNA, 5 µl of Taqman Gene Expression Master Mix, 0.5 µl of specific TaqMan Gene Expression Assay and 2.5 µl of nuclease free water. PCR cycling conditions were 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C each. The standards used for assay optimisation were prepared from cDNA generated from HTR8/SVneo trophoblast cells grown in standard culture conditions. The dilution range of the standards used were 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64, and the efficiencies of qPCR were 100% +/- 10%. Gel electrophoresis was undertaken to confirm the amplicon size and one band. PCR was performed for 4 reference genes (*GAPDH*, *GUSB*, *HPRT1* and *HMBS*) (Table 2.2), amplified in a reaction volume of 10 µl containing 2 µl of cDNA, 5 µl of SsoFast Evagreen Supermix (Bio-Rad, Hercules, CA, USA), 0.25 µl of forward primer (10 µM), 0.25 µl of reverse primer (10 µM) and 2.5 µl of nuclease free water. Cycling conditions for the reference genes were 30 sec at 95°C followed by 40 cycles of 5 sec at 95°C and 5 sec at 63°C each.

Duplicates of each sample were run in each experiment for all the experimental conditions. All qPCR were run on the CFX384 Touch Real-Time PCR Detection System (C1000 Touch Thermal Cycler) (Bio-Rad, Hercules, CA, USA) and qPCR data were viewed using CFX Manager Version 2.1 software. Expression of all reference genes and genes of interest were calculated using Cq (Quantification cycle) values and amplification efficiencies. Selected reference genes for normalisation were chosen based on reference gene expression stability as determined by the CFX Manager Software (Bio-Rad) and in accordance with Hellemans et al. 2007 [156] acceptable values for stably expressed reference genes. Using this approach *GUSB* and *HMBS* (mean CV value = 0.0551, mean M value = 0.1590) were selected for qPCR normalisation of *IGFBP3*, *IGFBP5*, *MMP1*, *VEGFA*, *P4HA1, P4HA2* and *ANGPTL4* and relative quantities were calculated using the ΔCq method. Calculation of normalised qPCR data (ΔΔCq) from raw qPCR data (Cq values) was based on the average Cq of replicates, conversion of Cq values into relative quantities based on the gene specific amplification efficiency, calculation of sample specific normalisation factor by taking the geometric mean of the relative quantities of the reference genes and finally the normalisation of quantities divided by the normalisation factor [156].

Table 2.1: List of genes selected for qPCR and assay ID

Gene	**Gene name**	**Assay ID**
IGFBP3	Insulin-like growth factor binding protein 3	Hs00426289_m1
IGFBP5	Insulin-like growth factor binding protein 5	Hs00181213_m1
MMP1	Matrix metallopeptidase 1	Hs00899658_m1
VEGFA	Vascular endothelial growth factor A	Hs00900055_m1
P4HA1	Prolyl 4-hydroxylase, alpha polypeptide I	Hs00914594_m1
P4HA2	Prolyl 4-hydroxylase, alpha polypeptide II	Hs00990001_m1
ANGPTL4	Angiopoietin-like 4	Hs01101125_m1

Table 2.2: List of reference genes and their primer sequences run in qPCR

Name	Sequence	Primer length	PCR product size
GAPDH-F2	CTCTCTGCTCCTCCTGTTCGAC	22	69 bp
GAPDH-R2	TGAGCGATGTGGCTCGGCT	19	
GUSB-F	CGTCCCACCTAGAATCTGCT	20	94 bp
GUSB-R	TTGCTCACAAAGGTCACAGG	20	
HPRT1-F	GTTATGGCGACCCGCAG	17	107 bp
HPRT1-R	ACCCTTTCCAAATCCTCAGC	20	
HMBS-F	CCACACACAGCCTACTTTCCAA	22	70 p
HMBS-R	TTTCTTCCGCCGTTGCA	17	

2.2.7 Statistical analysis

The normalised qPCR data were analysed using GraphPad Prism Version 6 software. ANOVA and Tukey's multiple comparison tests were performed to observe the comparison between multiple groups. Unpaired t-test was performed to compare the expression between 1% and 5% oxygen treatment. Statistical significance was defined as $P<0.05$. The fold change was calculated on the mean expression.

2.2.8 Protein extraction

Proteins were extracted from different oxygen treated HTR8/SVneo trophoblast cells using RIPA buffer (10 mM Tris, 100 nM NaCl, 1 mM EDTA and 1% Triton X-100, pH 7.4) and Complete Mini Protease Inhibitor (Roche, Mannheim, Germany) (1 tablet for 10 ml of RIPA buffer). RIPA buffer (74 µl) containing protease inhibitor was mixed with each pellet (collected from cell culture work) and centrifuged at 10,000 rpm for 10 min in 4ºC. The supernatant was transferred to a new tube and stored at -80ºC until used for western immunoblotting. The concentration of protein was measured by Bradford Assay in Nunclon™ Delta Surface 96 well Microtiter plate using Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (BSA, Sigma-Aldrich, St Louis, USA) (1 to 6 mg/ml) was used as a standard for this assay. Absorbance was taken at 595 nm on DYNEX TRIAD™ Series Multimode Detector

(DYNEX Technologies, Inc., Chantilly, VA, USA) using Concert TRIAD Series Multimode Detection software version 2.1.0.17. All the standards and samples were run in duplicate and the average of the absorbance was used to determine the protein concentration.

2.2.9 Western immunoblotting

Forty µg of protein from each sample was used for western immunoblotting. Samples were prepared to 30 µl with the inclusion of Laemmli 2x buffer (Bio-Rad, Hercules, CA, USA) and were heated at 95ºC for 5 min. Proteins were separated by SDS-PAGE using 10% Mini Protean TGX Gel (Bio-Rad, Hercules, CA, USA) at constant voltage, 150 volts and 400 mAMP for 1.5 h or until the tracking dye (Precision Plus protein Dual Color Standards) (Bio-Rad, Hercules, CA, USA) reached the bottom of the gel. After the separation, proteins were transferred onto PVDF membrane (Bio-Rad, Hercules, CA, USA). The run conditions for the transfer were constant mAMP, 150 V and 400 mAMP for 1.5 h at 4°C. The PVDF membrane was blocked with 5% skim milk in TBST (mixture of Tris-Buffered Saline and Tween 20) overnight at 4°C. Then the membrane was incubated with different primary antibodies (prepared in 5% skim milk-TBST) (Table 2.3) for 1 h at room temperature. The membrane was washed with TBST for 5 min (3 times) followed by the incubation with appropriate Horseradish peroxidase (HRP) conjugated secondary antibody (prepared in 5% skim milk-TBST). The membrane was further washed 3 times with TBST for 5 min each. From the blocking step onward, all the steps were performed with gentle agitation. Finally the antibody was detected using Amersham ECL reagent (GE Healthcare, Buckinghamshire, UK) and the image of the membrane was developed on Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK). Two prostate cancer cell lines (PC3 and LNCaP) and one ovarian cancer cell line (OVCAR) were used for the optimisation of the primary antibodies. Calnexin antibody was used as a loading control. Calnexin is an integral membrane protein of the endoplasmic reticulum

found throughout the eukaryotic kingdom. It appears to be present in most organisms and it acts as chaperone. In western blots, calnexin appears as 90 kDa band.

Table 2.3: List of antibodies used in western blotting

Primary Antibody	Catalog No	Host	Dilution	HRP conjugated secondary antibody	Dilution
Calnexin	MA3-027	Mouse	1:4000	Goat anti-mouse IgG1	1:1000
IGFBP3	sc-9028	Rabbit	1:1000	Goat anti-rabbit IgG	1:5000
MMP1	sc-21731	Mouse	1:100	Goat anti-mouse IgG1	1:1000
VEGFA	sc-152	Rabbit	1:500	Goat anti-rabbit IgG	1:5000
P4HA1	ab127564	Rabbit	1:500	Goat anti-rabbit IgG	1:5000

Note: sc=Santa Cruz Biotechnology, Inc, Dallas, Texas, USA, ab=Abcam, Cambridge, CB4 0FL, UK.

2.3 Results

2.3.1 Immunofluorescence of HTR8/SVneo cells

Immunofluorescence of HTR8/SVneo cells showed that HIF-1α is highly expressed in the nuclei of trophoblasts treated with 1% oxygen compared to 5% oxygen whereas expression of HIF-2α was unchanged by oxygen treatment (Figure 2.1).

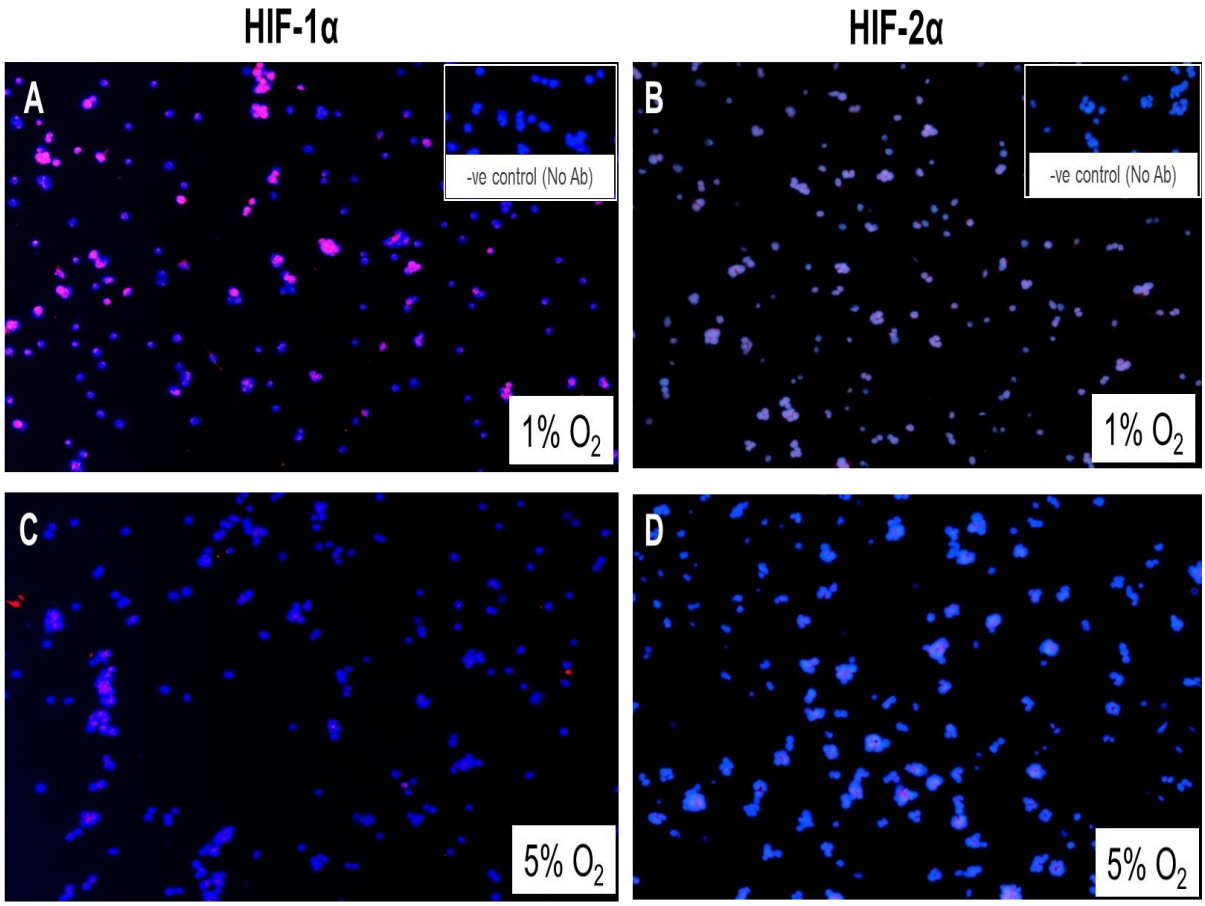

Figure 2.1: Localisation of HIFs in HTR8/SVneo cells in different oxygen environment
Localisation of HIF-1α was visible in the nucleus in 1% oxygen treated HTR8/SVneo trophoblast cells compared to 5% oxygen whereas localisation of HIF-2α was unchanged in different oxygen environments. Magnification = 20X, blue labelling = DAPI nuclear stain, red labelling = HIF positive stain.

2.3.2 HTR8/SVneo microarray

The microarray analysis showed differential expression of genes of HTR8/SVneo trophoblast cells incubated in different oxygen concentrations (1%, 5% and 20%). 290 genes were differentially expressed in 1% vs 5% oxygen treated HTR8/SVneo (Figure 2.2), 314 genes were differentially expressed in 1% vs 20% oxygen treated trophoblast cells. However, only 8 genes were differentially expressed in 5% vs 20% oxygen treated cells, providing evidence that standard culture conditions (20% oxygen) are not markedly different to more physiologically relevant conditions (5% oxygen) for this cell line. The 1% and 5% oxygen comparison better matched with early and late first trimester oxygen concentration changes within the first

trimester placenta and the genes differentially expressed in this comparison were further investigated (see below).

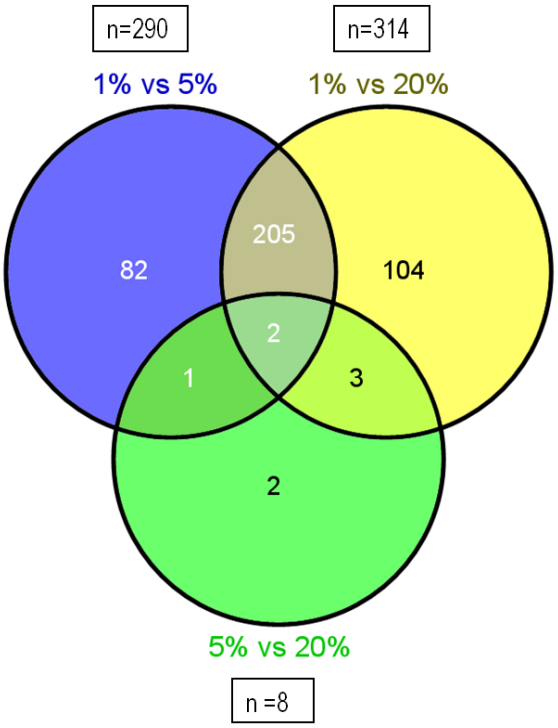

Figure 2.2: Venn diagram of differentially expressed genes in HTR8/SVneo cells
Venn diagram showing the overlap in differentially expressed genes in HTR8/SVneo cells cultured in different oxygen concentrations. 290, 314 and 8 genes are differentially expressed in 1% vs 5%, 1% vs 20% and 5% vs 20% oxygen, respectively. A fold difference of ≥1.5 and P<0.05 were used to define the differential expression of genes.

2.3.3 Ingenuity Pathway Analysis (IPA)

To determine the genes and pathways affected by differences in oxygen concentration, differentially expressed genes identified in the microarray analysis above were analysed by Ingenuity Pathway Analysis. IPA demonstrated that in the 1% vs 5% comparison group, HIF-1α was identified as the top upstream transcription regulator. The activation z-score for HIF-1α was 4.444 (activated when z-score is ≥2). That is, HIF-1α is predicted to regulate a number of genes in the dataset. IPA also showed that transcription of 41 genes is directly regulated by HIF-1α (Table 2.4) and among these 41 genes, the direction of change of expression of 31 genes was consistent with HIF-1α activity. Many of those 41 genes were involved in cell death & survival, cell growth & proliferation, cell cycle, cellular movement and tumor morphology. IPA

analysis of known interactions of these 41 genes revealed 3 networks. Networks 1, 2 and 3 include 16, 14 and 11 genes, respectively. Top functions of network 1 included Carbohydrate Metabolism, Small Molecule Biochemistry, Cardiovascular System Development and Function, those of network 2 (Figure 2.3) included Cellular Development, Cellular Growth and Proliferation, Skeletal and Muscular System Development and Function, and those of network 3 included Cell Morphology, Cellular Function and Maintenance, Cellular Compromise. From these 41 genes, *IGFBP3*, *IGFBP5*, *MMP1*, *VEGFA*, *P4HA1* and *P4HA2* were selected for validation by qPCR in an independent set of treated HTR8/SVneo samples. All of these genes, except for *MMP1* have HIF-1α:HIF-1β(ARNT) transcription factor binding sites. *IGFBP3*, *IGFBP5*, *MMP1* and *VEGFA* have previously been shown to be associated with trophoblast functions [157-165]. *P4HA1* and *P4HA2* are associated with HIF functions and have been shown to play a role in cellular invasion and metastasis [166, 167].

Table 2.4: List of 41 genes regulated by HIF-1α in 1% vs 5% oxygen comparison group

Genes in dataset	**Entrez Gene Name**	**Fold Change (1% vs 5%)**
ADM	Adrenomedullin	2.065
ALDOC	Aldolase C	1.492
ANGPTL4	Angiopoietin-like 4	8.168
BHLHE40	Basic helix-loop helix family, member e40	2.313
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	4.330
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	1.991
CA9	Carbonic anhydrase IX	1.324
CCND1	Cyclin D1	-1.262

CYR61	Cysteine-rich angiogenic inducer, 61	-1.316
EGLN1	Egl nine homolog 1 (C. elegans)	1.379
ENO2	Enolase 2	2.015
ERO1L	Endoplasmic reticulum oxidoreductin 1-like	1.279
FAM162A	Family with sequence similarity 162, member A	1.430
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	1.314
GBE1	Glucan (1,4-alpha-), branching enzyme 1	1.408
GPI	Glucose-6-phosphate isomerase	1.312
H2AFX	H2A histone family, member X	-1.275
HILPDA	Hypoxia inducible lipid droplet-associated	1.572
HK2	Hexokinase 2	3.109
IGFBP3	Insulin-like growth factor binding protein 3	2.925
IGFBP5	Insulin-like growth factor binding protein 5	-1.313
IL8	Interleukin 8	-1.500
KDM3A	Lysine (K)-specific demethylase 3A	1.995
LDHA	Lactate dehydrogenase A	1.452
MMP1	Matrix metallopeptidase I	-1.507
NDRG1	N-myc downstream regulated 1	4.215
P4HA1	Prolyl 4-hydroxylase, alpha polypeptide I	1.804
P4HA2	Prolyl 4-hydroxylase, alpha polypeptide II	1.447
PFKFB3	6-phosphofructo-2-kinase/fructose-	1.921

	2,6-biphosphatase 3	
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	5.424
PGK1	Phosphoglycerate kinase 1	1.769
PLAC8	Placenta-specific 8	1.433
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	1.650
PPFIA4	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4	2.789
RAB20	RAB 20, member RAS oncogene family	1.444
RASSF1	Ras association (RalGDS/AF-6 domain family member 1)	1.390
SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	2.232
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	2.279
STC2	Stanniocalcin 2	2.834
TPI1	Triosephosphate isomerase 1	1.372
VEGFA	Vascular endothelial growth factor A	1.261

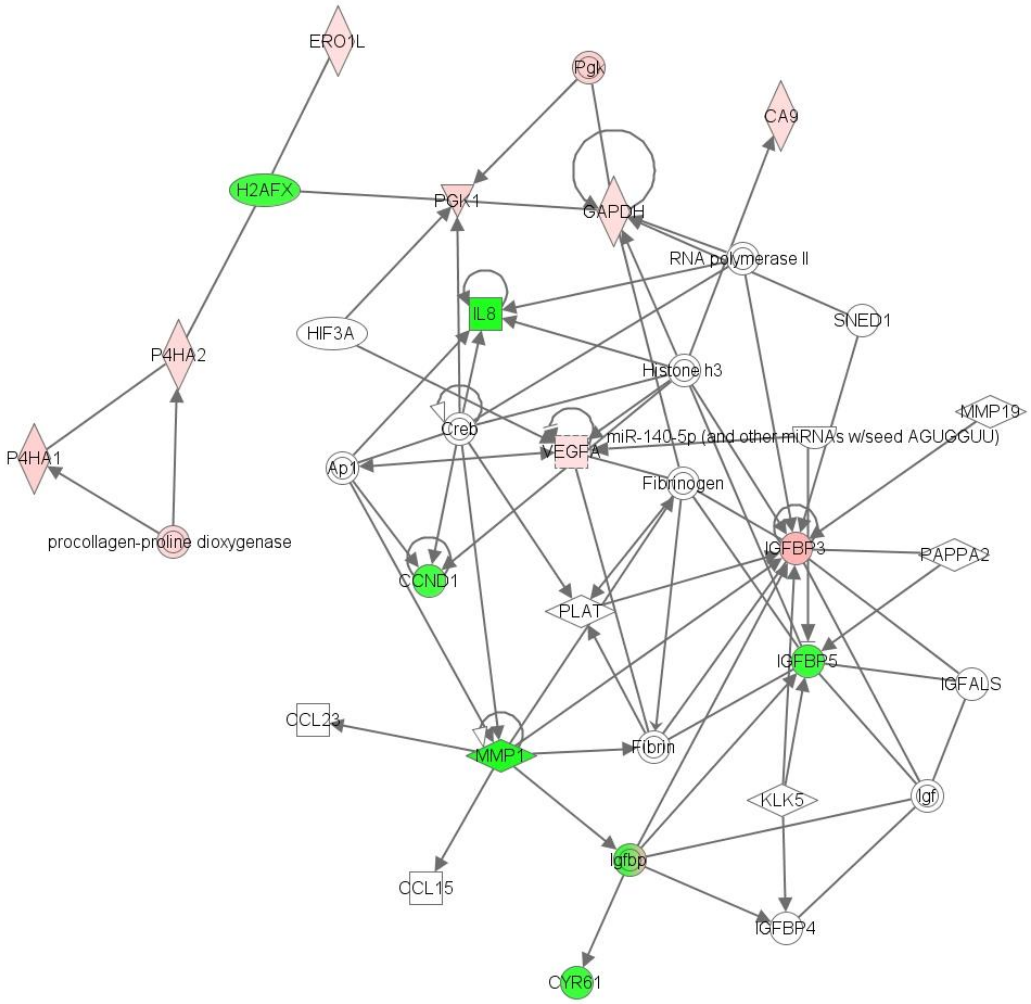

Figure 2.3: A gene-interaction network (network 2) between HIF-1α regulated genes
Top functions of this network include: Cellular Development, Cellular Growth and Proliferation, Skeletal and Muscular System Development and Function. The pink colour indicates genes up-regulated in 1% compared to 5% oxygen whereas the green colour indicates down-regulation. IGFBP3, VEGFA, P4HA1, P4HA2 were up-regulated in trophoblast cultured in 1% oxygen compared to 5%. IGFBP5, MMP1, IL8 were down-regulated in 1% oxygen treated trophoblast compared to 5%.

2.3.4 Analysis by Panther

Analysis by Panther Classification System displayed different molecular functions, biological processes, pathways, protein class and cellular component of the genes which have been found to be differentially expressed between 1% vs 5% oxygen treated HTR8/SVneo cells. A large proportion of these genes have binding (especially nucleic acid and protein binding) and catalytic activity (Figure 2.4).

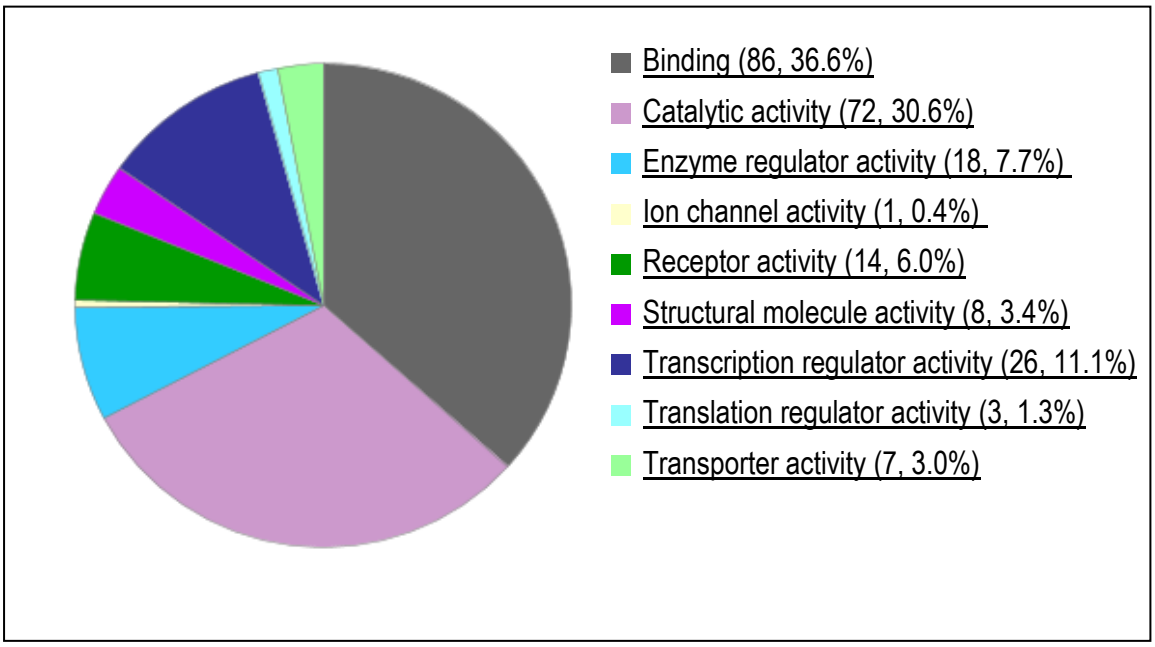

Figure 2.4: Molecular functions of differentially expressed genes
The number of genes, which were expressed differentially in 1% compared to 5% oxygen treatment in trophoblasts, and the percentage are shown in parentheses. A high percentage of genes are involved in binding and have catalytic activity

Panther analysis also indicated that the genes, which are differentially expressed in 1% vs 5% oxygen treated HTR8/SVneo cells, are associated with 48 pathways. A large number of these genes are involved in glycolysis, angiogenesis and signalling pathways (Figure 2.5).

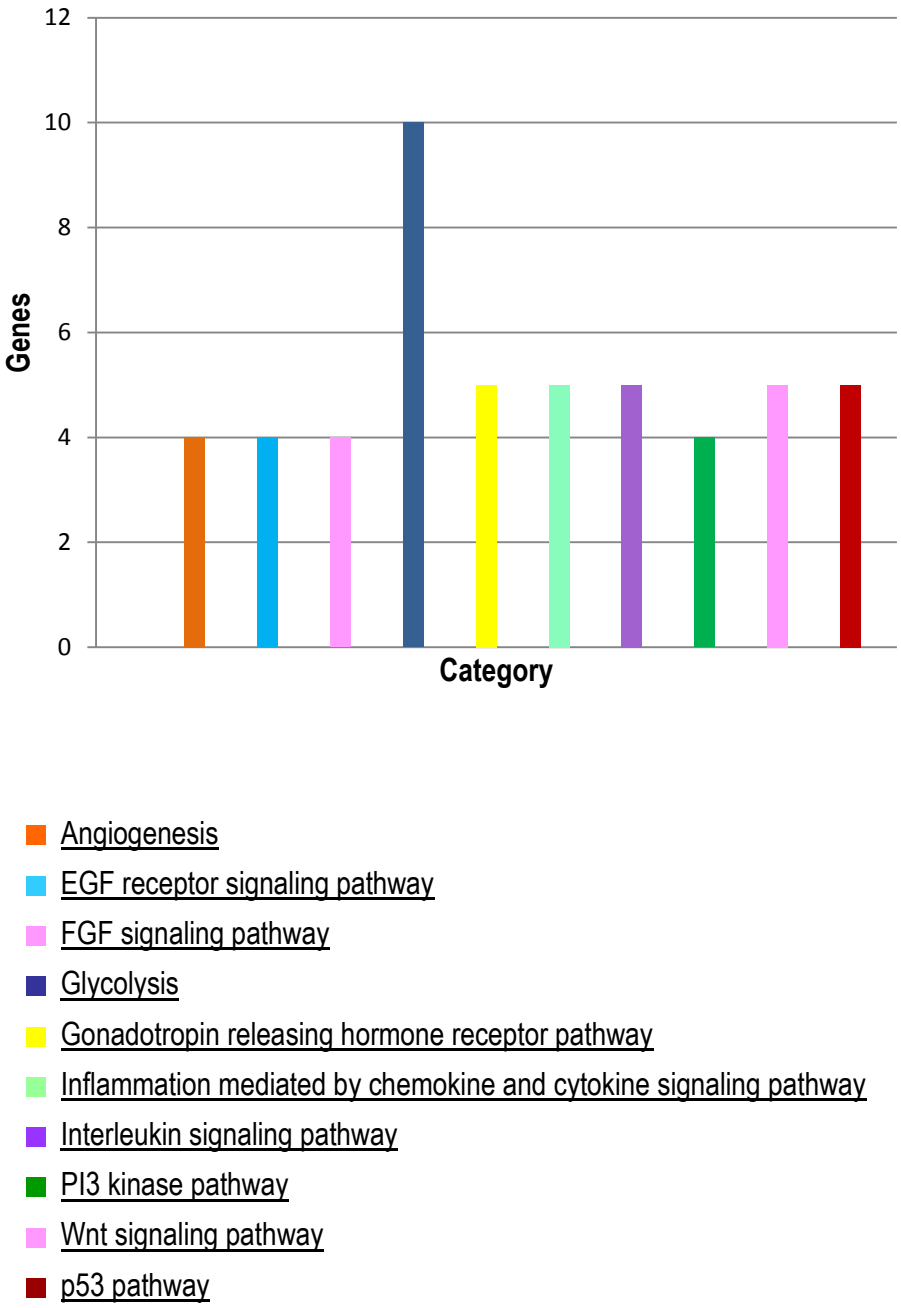

Figure 2.5: Pathways involved with the differentially expressed genes
Forty eight pathways have been identified to be involved with the genes which are differentially expressed between 1% vs 5% oxygen.

2.3.5 Quantitative PCR validation

Real time quantitative PCR (qPCR) was performed to validate the expression of genes selected from the microarray in an independent set of samples. qPCR data showed that the expression of *IGFBP3*, *VEGFA, P4HA1, P4HA2* and *ANGPTL4* were significantly higher in 1% compared to 5% and 20% oxygen treated HTR8/SVneo trophoblast cells (Figure 2.6) which is consistent with the microarray data (Table 2.5). Moreover, expression of *VEGFA* was also different between 5% and 20% oxygen treatment. However, this was not apparent in the microarray data. Significantly lower expression of *MMP1* was observed in 1% oxygen treated cells compared to 20%, but not in 5% oxygen. In addition, expression of *MMP1* was also different between 5% and 20% oxygen cultures. No significant difference was observed in the expression of *IGFBP5*.

Table 2.5: Fold change (1% vs 5%) and P value of genes from microarray and qPCR result

Gene name	Microarray		qPCR	
	Fold change	**P value**	**Fold change**	**P value**
IGFBP3	↑2.925	4.22E-06	↑4.637	<0.0001
IGFBP5	↓1.313	1.85E-04	↓1.413	0.0659
MMP1	↓1.507	4.21E-04	↓1.327	0.3715
VEGFA	↑1.261	2.74E-05	↑2.626	<0.0001
P4HA1	↑1.804	3.02E-06	↑3.297	0.0023
P4HA2	↑1.447	2.55E-05	↑1.261	0.0009
ANGPTL4	↑8.168	2.12E-09	↑35.8	0.0001

Note: P value of qPCR results were determined using unpaired t-test.

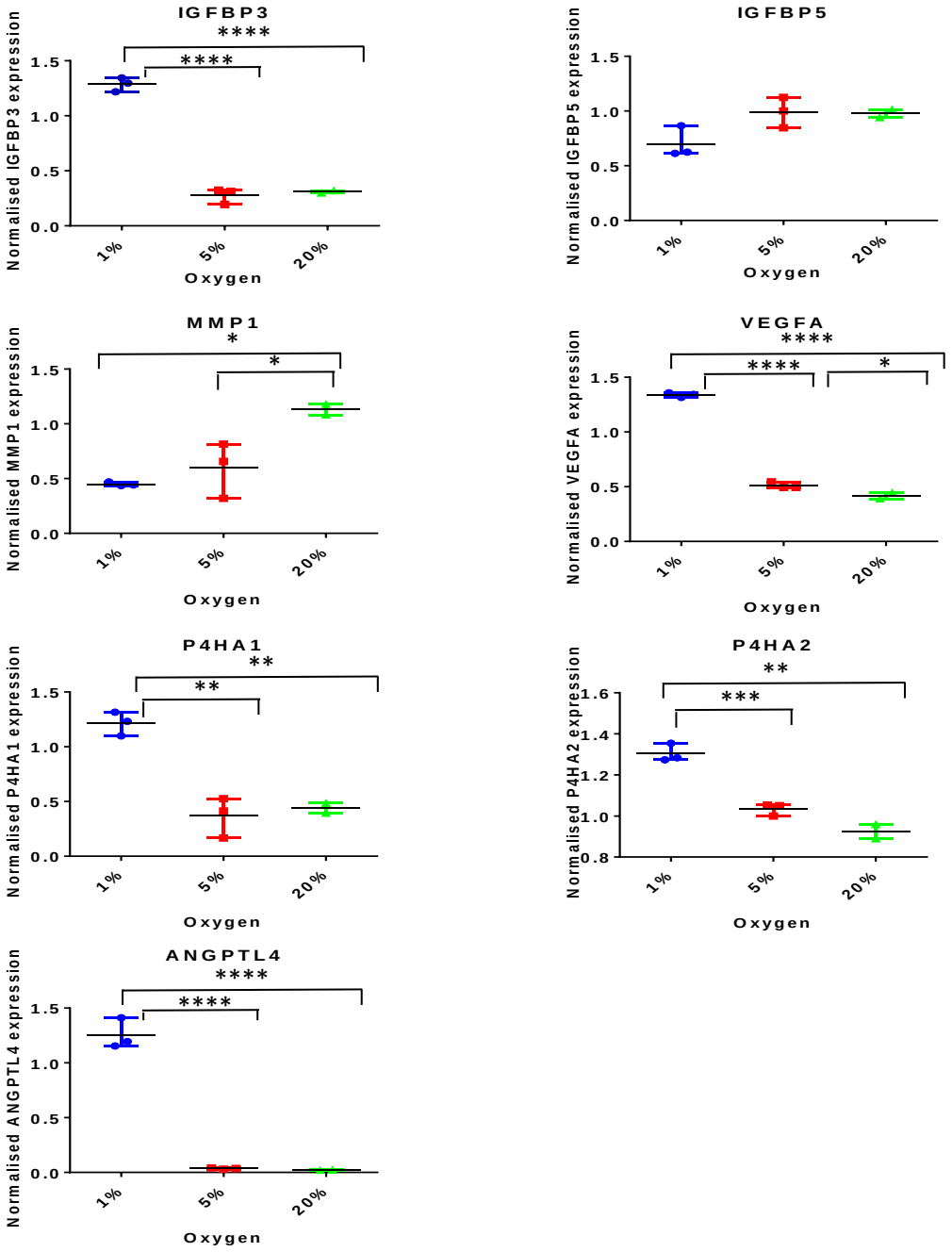

Figure 2.6: Quantitative PCR validation of selected genes of interest

*Expression of IGFBP3, IGFBP5, MMP1, VEGFA, P4HA1, P4HA2 and ANGPTL4 in HTR8/SVneo trophoblast cells treated with 1%, 5% and 20% oxygen. **** = P<0.0001, *** = P<0.001, ** = P<0.01 and * = P<0.05.*

2.3.6 Western immunoblotting

Antibodies were optimised using PC3, LNCaP and OVCAR cells based on previous reports. However, western immunoblotting on protein lysates from HTR8/SVneo cells was problematic most likely due to protein degradation though it was performed more than once (Figure 2.7). Further study is required to determine the expression of protein in HTR8/SVneo cells.

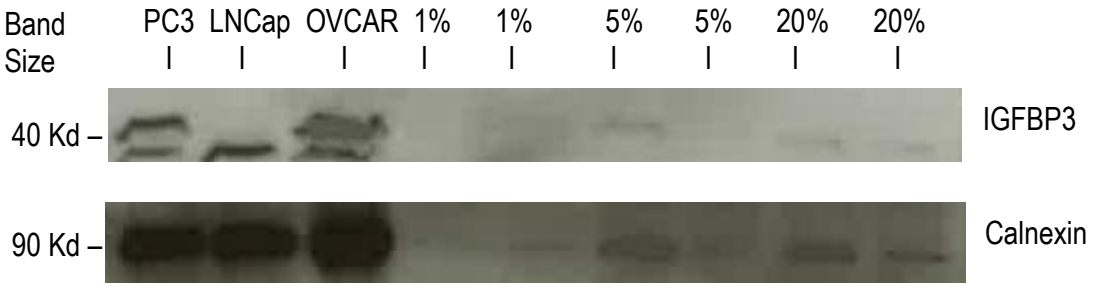

Figure 2.7: Western immunoblotting
Protein expression of IGFBP3 and Calnexin in PC3, LNCap, OVCAR and in HTR8/SVneo trophoblast cells treated with 1%, 5% and 20% oxygen.

2.4 Discussion

We have identified differential gene expression in HTR8/SVneo trophoblast cells cultured in different oxygen atmospheres. We assumed that 1% and 5% oxygen mimics the early first trimester and late first trimester placental oxygen environments. Early placental development occurs in a hypoxic condition and HIF-1α is stabilised in such an environment. We are considering two different levels of oxygen tension within the placenta during first trimester. Factors associated with trophoblast functions act differently in response to varied oxygen tension. We believe that oxygen, hypoxia and HIF-1α play a major role in this transition stage in the regulation of gene expression. IPA analysis of our microarray data showed that HIF-1α is the top upstream transcription regulator of the genes which are expressed differentially in trophoblasts treated in 1% vs 5% oxygen. So this finding along with increased nuclear localisation of HIF-1α protein in 1% oxygen compared to 5% is an expected result.

Both the microarray and qPCR results for all the genes of interest (except for *IGFBP5*) have shown a similar direction of expression change. Our study showed that expression of *IGFBP3* is increased in 1% oxygen compared to 5% and 20%. A similar study by Koklanaris et al. that used HTR8/SVeo cells, cultured for 6 hours at 37°C, also confirmed the upregulation of *IGFBP3* in 0-1% oxygen compared to normoxia (20% oxygen) [168]. The IGF system plays a crucial role in pregnancy through placental and fetal development [169]. IGFBP3, which is an insulin-antagonizing peptide, binds free IGFs with high affinity and increases their bioavailability. *IGFBP3* is expressed in invasive trophoblasts along with *IGF1* and especially high levels of *IGF2* [170]. So our result of increased level of *IGFBP3* expression in 1% oxygen may be associated with a role in trophoblast invasion during early first trimester.

MMP1 is a metallopeptidase with a role in extracellular matrix (ECM) degradation. MMP1 is highly expressed in a variety of cancer cells and involved in invasion and metastasis of cancer cells such as hepatocellular carcinoma (HCC) [171, 172], breast carcinoma [173], prostate cancer [174], colorectal cancer [175] and pancreatic cancer [176]. A study carried out in different HCC cell lines has shown that membrane palmitoylated protein 3 (MPP3) promotes migration and invasion through the up-regulation of *MMP1* [172]. Another very recent study conducted on different trophoblast cell lines including HTR8/SVneo has found that trophoblast migration and invasion is controlled by FOS transcription factors via *MMP1* [177]. That study showed that the induction of migration and invasion is associated with the increased expression of *MMP1*. Jiang et al. demonstrated that knockout of SPARC (secreted protein acidic and rich in cysteine) inhibits HTR8/SVneo tropboblast invasion accompanied by the down-regulation of MMP1 [178]. However, there is no study considering the effect of oxygen concentration on MMP1 expression in trophoblasts. In our study, expression of *MMP1* was significantly decreased in 1% oxygen treated trophoblasts compared to 20% but not 5%. As the

expression of *MMP1* was not different in 1% vs 5%, this suggests that it may not differ in early vs late first trimester. It would also be interesting to look at the protein level of MMP1 in trophoblasts in response to oxygen.

VEGFA expression was also increased in 1% oxygen compared to 5% and 20% oxygen. It is known that the VEGF family plays an essential role in angiogenesis [179], which is vital for differentiation of trophoblasts and maternal spiral artery remodelling [180], which is a consequence of trophoblast invasion. In one study, VEGF was shown to increase motility of SGHPL4 trophoblast [181]. Though VEGF did not stimulate invasion, it was suggested that VEGF could have role in attracting trophoblast cells to the decidua [181]. *VEGFA* is regulated by HIF-1α [182, 183]. In one study, hypoxia (1% oxygen) was shown to induce *VEGF* expression by 8-fold in isolated human term cytotrophoblasts and *in vitro* differentiated syncytiotrophoblast compared to 21% oxygen [184]. Expression of secreted VEGF has also been shown to be increased significantly in first trimester and term cytotrophoblasts cultured in 5% oxygen compared to 20% [185] A study conducted in Ras-transformed cells proposed that hypoxia induces *VEGF* expression mediated by activated PI3-kinase/Akt pathway and HIF-1 [186]. So our result suggests that low oxygen induces *VEGFA* expression which is likely associated with increased angiogenesis and maternal spiral artery remodelling in the early first trimester placenta.

Collagen prolyl-4 hydroxylases (P4Hs) catalyse the hydroxylation of a proline residue to form 4-hydroxyproline which is essential for collagen biogenesis required for ECM deposition [187, 188]. The ECM is essential for diverse cellular events such as adhesion, migration, proliferation, differentiation and survival [189, 190]. Increased *P4HA1* and *P4HA2* expression may coincide with the decreased *MMP1* expression in 1% oxygen treatment. In normoxic conditions, P4Hs catalyses the hydroxylation of HIF-1α followed by its degradation. However, P4HA1 and P4HA2 were shown to be increased by hypoxia in various cell types [191, 192]. A

study conducted in fibroblasts showed that HIF-1α accelerates ECM remodelling through inducing *P4HA1* and *P4HA2* [167]. In our study, increased levels of *P4HA1* and *P4HA2* expression in trophoblasts cultured in 1% oxygen may also associate with increased trophoblast invasion.

Expression of *ANGPTL4* was significantly increased in 1% oxygen atmospheres compared to 5% and 20%. *ANGPTL4* is induced by hypoxia and its expression has been shown to be mediated by HIF-1α in various cells [193-196]. ANGPTL4 has a role in angiogenesis [197, 198]. A study conducted in HCC cells showed that both mRNA and protein expression of ANGPTL4 was significantly increased in hypoxic conditions (2% oxygen) compared to normoxia when cultured for 24 hours and the overexpression of ANGPTL4 was directly upregulated by HIF-1α [199]. In addition, this overexpression of ANGPTL4 significantly increased the transendothelial migration of HCC cell *in vitro* and, intrahepatic and distal pulmonary metastasis *in vivo*. Their findings suggested that *ANGPTL4* is a target gene of HIF-1α and plays essential roles in metastasis of HCC cell [199]. So in our study, increased level of *ANGPTL4* in 1% oxygen treated trophoblasts is likely to be associated with increased angiogenesis during early first trimester of pregnancy.

2.5 Conclusion

Increased levels of HIF-1α protein localised to the nucleus of HTR8/SVneo trophoblast cells were found in 1% oxygen cultures. Differential gene expression in 1% vs 5% oxygen and the direction of change in expression of these genes is consistent with the translocation of HIF-1α. Specifically, increased expression of *IGFBP3*, *VEGFA*, *P4HA1, P4HA2* and *ANGPTL4 was* observed in 1% oxygen compared to 5% oxygen. These genes play important roles in trophoblast functions required for early placental development. Together our findings suggest that factors involved in early placental development are regulated by oxygen, and HIF-1α is likely to be the master regulator.

Chapter 3: Expression of hypoxia-responsive gene in first trimester, term control and term complicated placental tissue

3.1 Introduction

Placenta is a specialized organ which provides appropriate exchange of oxygen and nutrients between the fetus and the mother. Placental development is a dynamic process with predominance of trophoblast proliferation, invasion, differentiation and functions at different times across gestation. These trophoblast functions are regulated by a number of factors like oxygen, hypoxia and hypoxia inducible factors (HIFs). While the expression of *HIF1A* mRNA has been reported to remain unchanged throughout gestation, expression of *HIF2A* mRNA increases with gestational age [90]. In the same study, both HIF-1α and HIF-2α protein, but not mRNA decreased significantly with gestational age suggesting the regulation of HIFs at the protein level is different to that at the transcriptional level [90].

Although oxygen is essential for healthy fetal and placental development, very early fetal and placental development occurs in a hypoxic environment. This physiological hypoxia is essential for the development at this stage. There is no maternal blood flow to the intervillous space (IVS) of the placenta until the end of the first trimester (approximately 11-12 weeks of gestation) [64, 65]. Prior to this the endovascular cytotrophoblasts (CTB) initially invade and plug the maternal spiral artery resulting in limited or no blood supply to the intervillous space (IVS). At the end of first trimester, the plugs are displaced and maternal blood starts to flow into the placenta [55]. Insufficient trophoblast invasion results in poor spiral artery remodelling and precedes major pregnancy complications like preeclampsia (PE), gestational hypertension (GH), intrauterine growth restriction (IUGR), preterm birth (PTB) and miscarriage [13, 23, 62, 200-203].

Hypoxia is a critical factor in normal fetal development, as well as in pregnancy complications. Consequently, HIF-1α and HIF-2α are of major interest as these factors respond differently with the

change of oxygen environment across gestation [90]. A number of findings have suggested that expression of HIF-1α and HIF-2α proteins are altered in preeclamptic placenta compared to that in uncomplicated pregnancies [31, 123, 124]. One of these studies suggested that HIF-1α protein is overexpressed in preeclamptic placenta and can bind to the hypoxia response element (HRE) of DNA, and also is able to regulate genes which are involved in angiogenesis [124]. However, how oxygen and hypoxia regulate placentation and what other factors are involved with the regulation caused by HIFs still need to be clarified.

We hypothesise that regulation of genes, which are involved in trophoblast functions, in healthy as well as complicated pregnancy are mediated by HIFs. In this study, we will determine the localisation and the level of expression of HIF-1α and HIF-2α protein at different time points in first trimester placenta and term control placenta. In addition, we will also examine differential expression of hypoxia responsive genes and their proteins in first trimester and complicated term placenta compared to term control placenta. Our study will further assist us to understand the regulation of hypoxia-responsive genes mediated by HIFs within the placenta during different stages of gestation.

3.2 Materials and Methods

3.2.1 Placental sample collection

Human first trimester placenta samples were collected from pregnant women undergoing elective pregnancy termination at the gynaecology clinic of the Women's and Children's Hospital, Adelaide, South Australia with ethics approval granted from the Children Youth and Women's Health Service Research Ethics Committee (REC1835/8/09). Term placentas from elective caesarean sections were collected from Lyell McEwin Health Service, Elizabeth Vale, South Australia with ethics approval from the SA Health Human Research Ethics Committee (HREC/12/TQEHLMH/16).

3.2.2 Sample processing

Placental samples were divided into five groups: (1) early first trimester (6 to 8 weeks of gestation), (2) late first trimester (10 to 12 weeks of gestation), (3) term uncomplicated, (4) term complicated with gestational hypertension (GH, high maternal blood pressure in the absence of proteinuria) and (5) term complicated with preeclampsia (PE). As apparently there is no maternal blood flow to the placenta before 9 weeks of gestation, this time was selected as the threshold between early and late first trimester. First trimester placental tissues were collected immediately after surgical termination and the term placental tissues were collected immediately after delivery. A full thickness sample of approximately 0.5 x 0.5 cm was collected from each term placenta and approximately 100 mg pieces were dissected from first trimester placenta. First trimester and term decidual and villous tissues were washed in sterile PBS and fixed in 10% neutral buffered formalin for 48 hours at 4ºC. Samples were washed four times with 1X PBS and stored in 70% ethanol at 4ºC before being embedded in paraffin blocks and cut into 5 µm sections for histology and immunohistochemistry. For RNA extraction and qPCR, a portion of placental tissue was collected into RNAlater, stored at 4°C for 24h and then frozen at -80°C.

3.2.3 Histology and immunohistochemistry

To identify different cell types, sections of each placental sample were stained with haematoxylin (Sigma-Aldrich, St Louis, USA) and eosin (Sigma-Aldrich, St Louis, USA) and were observed under an Olympus microscope (Model CX31RBSF, Olympus Corporation, Tokyo, Japan). Immunohistochemistry was performed to localise HIF-1α and HIF-2α protein on 12 early first trimester (< 9 weeks of gestation), 11 late first trimester (> 9 weeks of gestation) and 10 term control placenta samples. After deparaffinisation, antigen retrieval was performed by boiling the slides with citrate buffer (1.05g citric acid + 500ml MQ H_2O, pH 6.5) on high in a Sanyo EM-2613 microwave (Sanyo electric Co., Singapore) for 5 min and at simmer for 15 min. Then the slides were left at room temperature to cool. For quenching endogenous peroxidases, the sections were incubated with 3% H_2O_2 for 1 h. The sections

were blocked with diluent (1% BSA and 10% pig serum in 1X PBS) for 1 h. Then sections were labelled with HIF-1α and HIF-2α (Novus Biologicals, Littleton, CO, USA) primary antibody (Table 2.1) and diluent as negative control followed by overnight incubation at room temperature in a humidified chamber. On the following day, the sections were incubated with goat anti rabbit secondary antibody (Dako Cytomation, Glostrup, Denmark) for 1 h followed by incubation with streptavidin-HRP conjugate (1:500, Rockland, Pennsylvania, USA) for 1 h at room temperature. All washes were performed in MQ H_2O and 1X PBS (3 times) for 5 min each. The site of antibody binding was detected using diaminobenzidine (Sigma-Aldrich, St Louis, USA) for 5-10 min. The appropriate brown colour was observed under the microscope. The tissue sections were counterstained with haematoxylin (Sigma-Aldrich, St Louis, USA) and then dehydrated and mounted with DPX mounting media (Sigma-Aldrich, St Louis, USA).

Table 3.1: List of primary and secondary antibodies used in immunohistochemistry

1° Ab	Catalog No	Host	Dilution	2° Ab	Dilution
HIF-1α	NB100-134	Rabbit	1:1500	Goat anti-rabbit	1:500
HIF-2α	NB100-122	Rabbit	1:1500	Goat anti-rabbit	1:500

3.2.4 Quantification of immunohistochemical staining

After immunohistochemical staining, images of tissue sections were captured by NanoZoomer Digital Pathology scanner (Model C9600 v1.2, Hamamatsu Photonics K.K.) using NDP Scan software (v2.2, Hamamatsu Photonics K.K.) at a magnification of 40X. The images were viewed using NDP View software (v2.2, Hamamatsu Photonics K.K.). From these images, 10 non-overlapping images were selected at random (at magnification of 40X) for scoring. The images were scored using a video image analysis system (VideoPro 32, Leading Edge, Marion, SA, Australia). Measurements of video images were performed on the positively stained (diaminobenzidine-stained) area, the total cell area (positively and negatively stained area) and the integrated optical density of the positively stained area of trophoblasts and villous stromal cells separately. For each image selection, the VIA positivity (%

positive stained area), the mean integrated optical density (MIOD, total amount of staining, average intensity of immunoreactivity per unit area = concentration, arbitrary units) and the mean optical density (MOD, intensity of staining) were determined (methods described in [204]). The average positivity, MIOD, MOD of 10 images per sample was used for data analysis. The appropriate thresholds for intensity of immunostaining were set for each individual antibody and staining run. Scoring of the total cells was obtained by adding the scores of trophoblasts and stromal cells together.

3.2.5 RNA extraction from placenta

Placental RNA was extracted from 11 early first trimester samples, 7 late first trimester samples, 10 term control samples, 8 term samples complicated with Gestational Hypertension and 12 term samples complicated with PE. Approximately 100mg of villous tissue was homogenised by the PowerLyzer 24 (MoBio Laboratories, Carlsbad, CA, USA) with ceramic beads (GeneWorks) at 3500 rpm for 30 seconds, twice. RNA was extracted using TRIzol reagent (Ambion**,** Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was confirmed by agarose gel electrophoresis and the concentration was determined using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE)**.** RNA integrity was confirmed using the Experion Station (Bio-Rad, Hercules, CA, USA).

3.2.6 Reverse transcription, quantitative PCR (qPCR)

RNA (4 µg) was treated with TURBO DNA-free™ DNase treatment kit (Ambion, Carlsbad, CA, USA) to remove DNA according to the manufacturer's protocol. A PCR was run using Faststart Universal Master Mix (Roche, Mannheim, Germany) and genomic DNA (gDNA) primers (IGF2R promoter region; F-GCCTCTTCTTGTTAATTTCCCTGTT, R- TTCAGTTTCTCCACAGACATTCAA, of 95bp amplicon length) [155] to check for gDNA contamination. Absence of gDNA was confirmed with the absence of amplification of DNase treated RNA. Cycling conditions were: 95°C for 10 min, then 40 cycles of 98°C for 5 sec and 60°C for 20 sec.

Reverse transcription of DNase treated RNA was performed using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. The resultant cDNA was

diluted 1:4 with nuclease free water. With this diluted cDNA, qPCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA) and specific TaqMan Gene Expression Assays (Table 3.2) (Applied Biosystems, Carlsbad, CA, USA) to validate the expression of genes of interest selected from microarray data (described in Materials and Methods section of Chapter 2) . All qPCR amplification reactions were run on the CFX384 Touch Real-Time PCR Detection System (C1000 Touch Thermal Cycler) (Bio-Rad, Hercules, CA, USA) and qPCR data were viewed using CFX Manager Version 2.1 software. The PCR reaction volume was 10 µl containing 2 µl of cDNA, 5 µl of TaqMan Gene Expression Master Mix, 0.5 µl of specific TaqMan Gene Expression Assay and 2.5 µl of nuclease free water. PCR cycling conditions were 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C each. Separate qPCR was performed for 4 reference genes (*GAPDH*, *GUSB, HPRT1* and *HMBS*) (Table 3.3). *GAPDH* primer sequences were previously published [205]. *GUSB* and *HPRT1* primer sequences were from qPrimerDepot (http://primerdepot.nci.nih.gov/), and *HMBS* primer sequences were from RTPrimerDB (http://medgen.ugent.be/rtprimerdb/) ID: 2953**.** Reference genes, previously optimised in the laboratory, were amplified in a reaction volume of 10 µl containing 2 µl of cDNA, 5 µl of SsoFast Evagreen Supermix (Bio-Rad, Hercules, CA, USA), 0.25 µl of forward primer (10 µM), 0.25 µl of reverse primer (10 µM) and 2.5 µl of nuclease free water. Cycling conditions for the reference genes were 30 sec at 95°C followed by 40 cycles of 5 sec at 95°C and 5 sec at 63°C each. Duplicates of each sample were run in each experiment for all the experimental conditions**.** Expression of all reference genes and genes of interest were calculated using Cq (quantification cycle) values and amplification efficiencies. Selection of reference genes were performed based on reference gene expression stability as determined by the CFX Manager Software (Bio-Rad) and in accordance with Hellemans et al. 2007 [156] acceptable values for stably expressed reference genes. Using this approach *GAPDH and HMBS* (mean CV value = 0.2038, mean M value = 0.5880) were selected for qPCR normalisation of *IGFBP3*, *IGFBP5*, *MMP1*, *VEGFA*, *P4HA1, P4HA2 and ANGPTL4* of early and late first trimester versus term control placenta group. *GAPDH* and *GUSB* (mean CV value = 0.2558,

mean M value = 0.7387) were selected for term control versus gestational hypertensive (GH) and preeclamptic (PE) placenta groups. Relative quantities were calculated using the ΔCq method. Calculation of normalised qPCR data (ΔΔCq) from raw qPCR data (Cq values) was based on the average Cq of replicates, conversion of Cq values into relative quantities based on the gene specific amplification efficiency, calculation of sample specific normalisation factor by taking the geometric mean of the relative quantities of the reference genes and finally the normalisation of quantities divided by the normalisation factor [156].

Table 3.2: List of TaqMan Gene Expression Assays and their assay ID.

Gene	Gene name	Assay ID
IGFBP3	Insulin-like growth factor binding protein 3	Hs00426289_m1
IGFBP5	Insulin-like growth factor binding protein 5	Hs00181213_m1
MMP1	Matrix metallopeptidase 1	Hs00899658_m1
VEGFA	Vascular endothelial growth factor A	Hs00900055_m1
P4HA1	Prolyl 4-hydroxylase, alpha polypeptide I	Hs00914594_m1
P4HA2	Prolyl 4-hydroxylase, alpha polypeptide II	Hs00990001_m1
ANGPTL4	Angiopoietin-like 4	Hs01101125_m1

Table 3.3: List of reference genes and their primer sequences

Name	Sequence	Primer length	PCR product size
GAPDH-F2	CTCTCTGCTCCTCCTGTTCGAC	22	69 bp
GAPDH-R2	TGAGCGATGTGGCTCGGCT	19	
GUSB-F	CGTCCCACCTAGAATCTGCT	20	94 bp
GUSB-R	TTGCTCACAAAGGTCACAGG	20	
HPRT1-F	GTTATGGCGACCCGCAG	17	107 bp
HPRT1-R	ACCCTTTCCAAATCCTCAGC	20	
HMBS-F	CCACACACAGCCTACTTTCCAA	22	70 p
HMBS-R	TTTCTTCCGCCGTTGCA	17	

3.2.7 Statistical analysis

Statistical significance was defined as $P<0.05$. The statistical analysis of the scoring of immunohistochemical staining was performed using IBM SPSS software version 21. These data were analysed by Independent-Samples T-test. The data are presented as mean ± SEM (Standard Error of the Mean). The normalised qPCR data were analysed using GraphPad Prism Version 6 software. ANOVA and Tukey's multiple comparison tests were performed to observe the comparison between multiple groups. Unpaired t-test was performed to compare the expression of genes of interest between first trimester and term placenta. The fold change was calculated on the mean expression.

3.3 Results

3.3.1 Immunohistochemistry

Immunohistochemical staining showed that HIF-1α and HIF-2α were localised to the nucleus and cytoplasm of invasive extravillous cytotrophoblast (EVT), syncytiotrophoblast (STB), cytotrophoblast (CTB) and villous stroma (VS) of early and late first trimester placenta with an increased nuclear expression in early first trimester. No expression of HIF-1α and HIF-2α was observed in term control placenta (Figure 3.1) which probably indicates a well oxygenated environment in this tissue. MOD (intensity of staining) scoring of the immunostaining showed significantly decreased expression of HIF-1α in the villous stroma (-5%, *P=0.001) and in the total cells (-4%, *P=0.013) of late first trimester placenta compared to early (Figure 3.2 C). However MIOD (total amount of staining) and positivity (% positive stained area) scoring showed that expression of HIF-2α was significantly increased in the trophoblasts (MIOD: +57%, *P<0.001 and Positivity: +45%, *P<0.001) and in the total cells (MIOD: +41%, *P<0.001 and Positivity: +35%, *P=0.001) of early first trimester placenta compared to late (Figure 3.2 B and F).

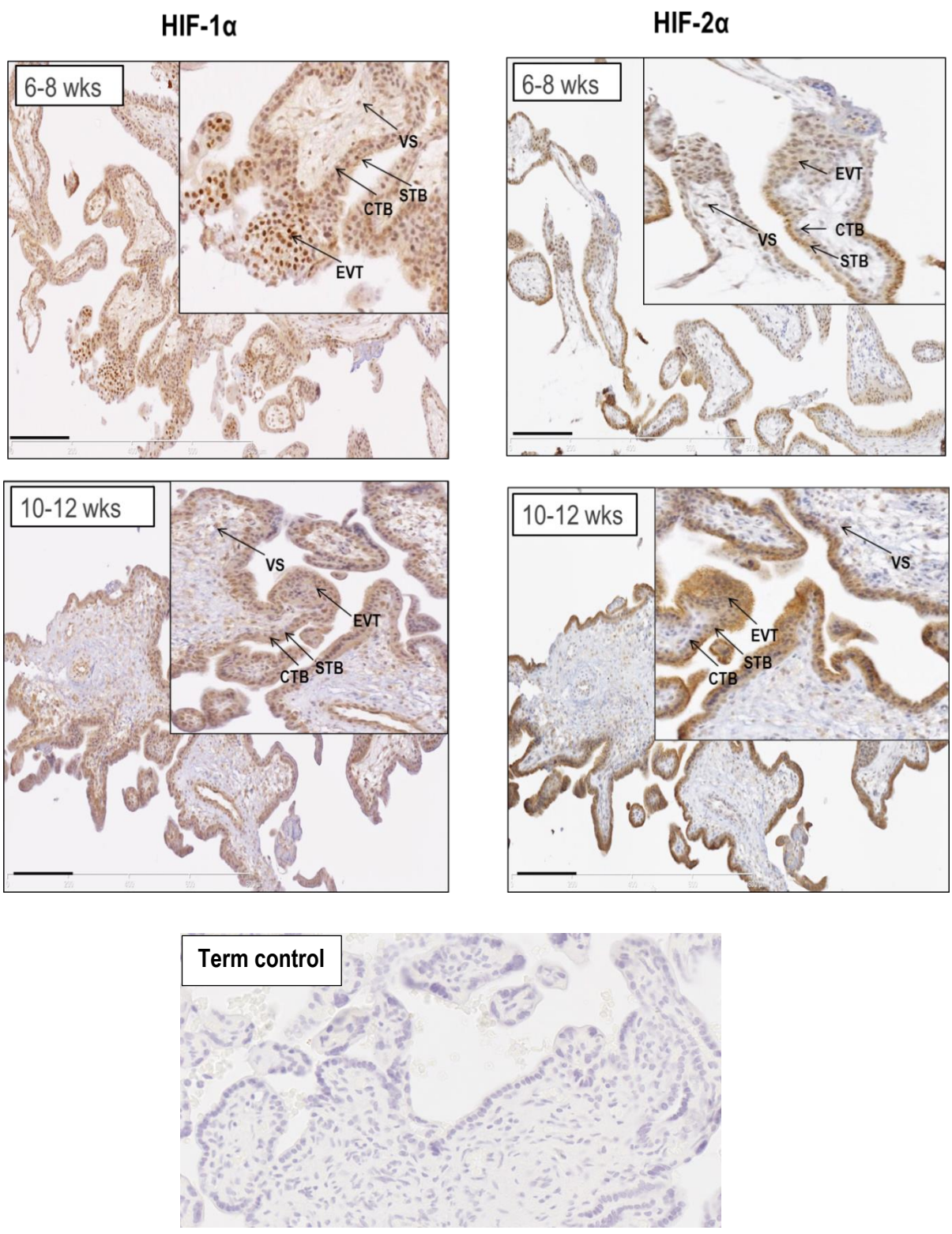

Figure 3.1: Localisation of HIF-1α and HIF-2α in early and late first trimester and term control placenta.

The arrows indicate the expression of HIF-1α and HIF-2α protein. The scale bar is 200 µm and the magnification is 20X.

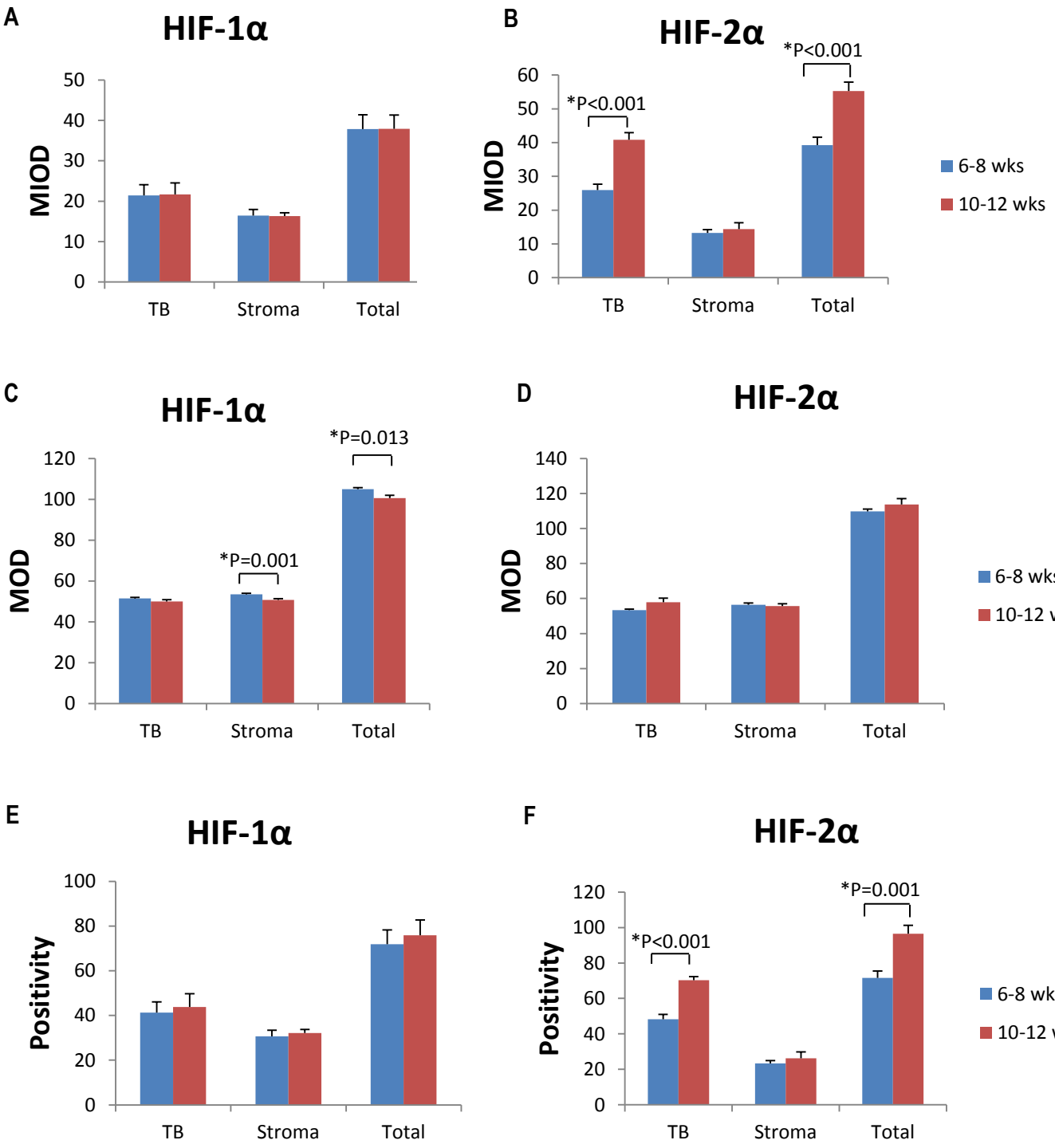

Figure 3.2: Immunoreactivity of HIF-1α (A, C and E) and HIF-2α (B, D and F) antibody in different regions of early and late first trimester placenta.

Expression of HIF-1α was significantly decreased in the stromal cells and in the total cells of late first trimester placenta compared to early (C). Expression of HIF-2α in the trophoblasts and in the total cells was significantly increased in late first trimester placenta compared to early. MIOD, MOD and Positivity are arbitrary units, TB = Cytotrophoblast, Syncytiotrophoblast and Extravillous trophoblast, Stroma = Villous Stroma, Total = TB +Stroma

3.3.2 Quantitative PCR

The qPCR was performed to assay the expression of IGFBP3, IGFBP5, MMP1, VEGFA, P4HA1, P4HA2 and ANGPTL4. The expression of IGFBP3, VEGFA, P4HA1, P4HA2 and ANGPTL4 were significantly higher in 1% compared to 5% oxygen treated HTR8/SVneo trophoblast cells (Result section of Chapter 2). Analysis of the qPCR data showed that the expression of IGFBP3 (*P<0.0001), IGFBP5 (*P<0.0001) and ANGPTL4 (*P<0.0001) were significantly higher in term control placenta compared to both early and late first trimester (Figure 3.3). The expression of *VEGFA* ((*P<0.0195) was significantly increased in term control placenta compared to early first trimester only (Figure 3.3). However, there was no difference in the expression of any of the genes between early and late first trimester placenta. So results of early and late first trimester placenta were combined together and compared to term control to obtain the P value and fold change (Table 3.4). Surprisingly, no difference in expression of any of the genes was observed in the term control placenta compared to term complicated (Figure 3.4). Expression of *MMP1* was very low to quantify accurately. So it was excluded from all the analyses.

Table 3.4: Differential gene expression in first trimester placenta vs term control using unpaired t-test

Gene name	P value	Fold Change
IGFBP3	<0.0001	↑2.2805
IGFBP5	<0.0001	↑6.1513
VEGFA	0.0195	↑2.3288
P4HA1	0.0519	↑1.6167
P4HA2	0.2338	↑1.2452
ANGPTL4	<0.0001	↑2.4406

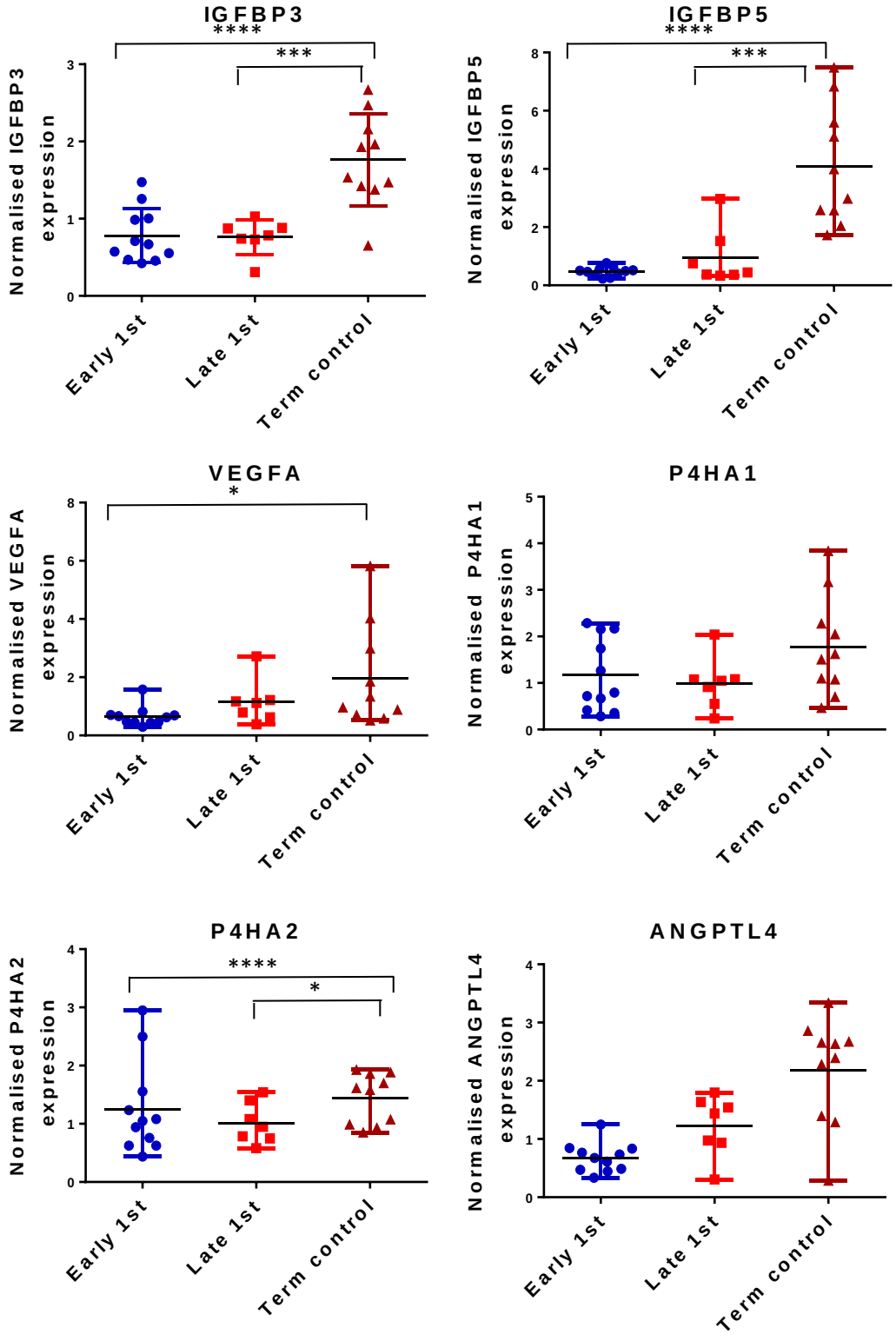

Figure 3.3: Expression of genes of interest in early and late first trimester placentas compared to term control

*Expression of IGFBP3, IGFBP5 and ANGPTL4 was significantly increased in term control placentas compared to either early or late first trimester. Expression of VEGFA was increased in term control placenta compared to early first trimester. **** = P<0.0001, *** = P<0.001 and * = P<0.05.*

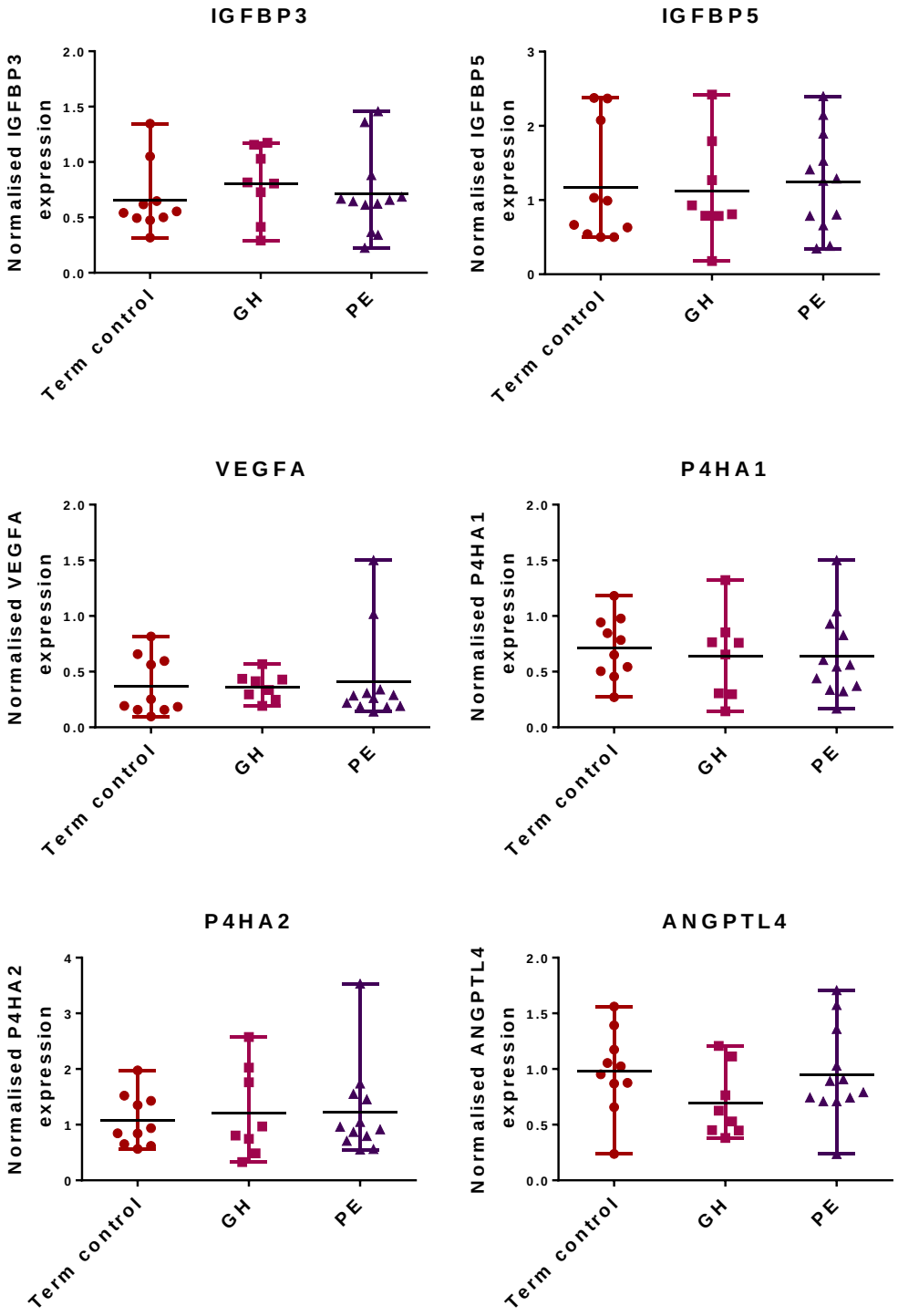

Figure 3.4: Expression of genes of interest in term complicated placentas compared to term control

No selected hypoxia responsive gene of interest (IGFBP3, IGFBP5, VEGFA, P4HA1, P4HA2 and ANGPTL4) was expressed differentially between these groups. GH and PE indicate gestational hypertension and preeclampsia, respectively.

3.4 Discussion

We hypothesised that the localisation and expression of HIF-1α and HIF-2α proteins differ between early and late first trimester placenta as there is a sharp change in oxygen concentration between those periods. Our data show differential localisation of HIF-1α and HIF-2α proteins in early and late first trimester placenta. During hypoxia (<5% oxygen), HIF-1α and HIF-2α are stabilised and translocate to the nucleus and dimerise with HIF-1β, forming active HIF-1 and HIF-2 which then bind to the hypoxia response element (HRE) of target genes to mediate transcription [59, 77, 88]. So our findings, increased nuclear localisation of HIF proteins and significantly increased expression of HIF-1α protein during early first trimester suggest increased HIF activity at that time. Previously, Rajakumar and Conrad have reported differential expression of HIFs in the placenta during different stages of gestation where they also suggested that expression of HIFs protein is increased by hypoxia in the placenta during early gestation [90]. So our findings are consistent with the literature.

Both HIF-1α and HIF-2α were highly expressed in invasive extravillous cytotrophoblast (EVT) in cell columns of early first trimester placenta. These EVT from the distal end of the column, invade and migrate into the maternal decidua. So it is likely that hypoxia responsive genes are also involved in trophoblast invasion. Although HIF-1α and HIF-2α regulate many similar genes, expression patterns of HIF-1α and HIF-2α are distinct likely due to the interactions with their different regulatory binding protein histone deacetylase 7 (HDAC7) and translation initiation factor 6 (Int6), respectively [80]. In a study conducted with neuroblastoma cells, HIF-2α has been shown to be active at 5% oxygen (similar to end capillary oxygen tensions) whereas HIF-1α was active at 1% oxygen [206]. This study also showed that knockdown of HF-2α reduces *VEGF* transcription at 5% oxygen whereas silencing of HIF-1α does not affect the expression of *VEGF* suggesting the ability of HIF-2α to regulate hypoxia responsive genes at physiological oxygen tension [207]. So the higher expression of HIF-2α in late first trimester with the expression of both HIFs during the first trimester could prolong transcription of

hypoxia responsive genes, which are essential for placental development and vascularisation through perfusion and oxygenation from the onset of maternal blood flow.

We have demonstrated differential expression of *IGFBP3, IGFBP5, VEGFA* and *ANGPTL4* in placenta of different gestations. All of these genes have binding site for HIF1α. Members of the IGF family are expressed in the placenta throughout pregnancy and play essential roles in placental and fetal development [169]. *IGFBP3* is expressed in invasive trophoblasts along with *IGF1* and especially high levels of *IGF2* [170]. Members of the VEGF family are expressed in the placenta throughout pregnancy and play important roles in placental angiogenesis, vascularization [208] and spiral artery remodelling [209, 210]. ANGPTL4 has a role in angiogenesis [197] and, along with VEGFA, has been shown to induce angiogenesis in first trimester placental trophoblasts [211]. As both HIF-1α and HIF-2α share many target genes, the increased expression of hypoxia responsive genes in term placenta probably coincides with the higher HIF-2α protein expression. A study conducted in breast cancer cell line reported the list of HIF-1α and HIF-2α binding sites using ChIP-seq, where lots of HIF-1α and HIF-2α binding sites are similar [212]. Interestingly that study found that both of HIF-1α and HIF-2α have binding sites for *IGFPB3* and *P4HA1*.

None of the genes assessed were expressed differentially between early and late first trimester placenta. This is likely consistent with the presence of both HIF-1α and HIF-2α proteins in both early and late first trimester. However, increased expression of *IGFBP3, IGFBP5, VEGFA and ANGPTL4* was observed in term placenta compared to first trimester which suggests expression of hypoxia responsive genes changes with the change in oxygen concentrations from first trimester to term. However, exposure to atmospheric oxygen levels during placental collection may have altered gene expression rapidly such that the expression profiles for these two phases of first trimester development appeared to be similar when perhaps they are not. This is a limitation of our collection system.

No difference was observed in the expression of any hypoxia responsive genes of interest in the term placenta complicated by either GH or PE compared to term control. It is widely assumed that the oxygen tension of preeclamptic placenta is lower due to poor trophoblast invasion and spiral artery remodelling. A global gene expression study conducted with 11 preeclamptic placenta concluded that the preeclamptic placentas are hypoxic [213]. However, the actual oxygen tension of preeclamptic placenta has not been measured. Huppertz *et al.* have actually suggested that oxygen tension is increased, rather than decreased, in preeclamptic placenta due to higher blood flow velocity caused by failure of spiral artery transformation [214]. So similar expression of hypoxia responsive genes in term control and complicated placenta suggests that oxygen levels in term control and complicated placentas are not different.

3.5 Conclusion

We expected increased HIF localisation observed in first trimester placenta would correlate with higher expression of hypoxia responsive genes in first trimester compared with term. Surprisingly expression of some hypoxia responsive genes was higher at term. This will require further investigation. Similar expression of hypoxia responsive genes in term complicated placenta compared to term control suggests similar oxygen atmosphere in both uncomplicated and complicated placenta, and that placental hypoxia might not be a feature of these complications at term. However, the protein level of the selected genes of interest across gestation and how the genes influence trophoblast functions are still to be elucidated. Future work could explain in what way all of these regulators are coordinated.

Chapter 4: Discussion

4.1 General Discussion

Appropriate trophoblast invasion through proper maternal spiral artery remodelling is vital for oxygenation of the placenta and fetus. A hypoxic environment is essential for early placental and fetal development. However, hypoxia later in pregnancy is associated with many complications. In the current study, the hypotheses were that (1) differential oxygen tension directed expression of hypoxia inducible factors (HIFs) and up-regulated the expression of hypoxia responsive genes essential for trophoblast functions, and (2) levels of HIF, and hypoxia responsive genes, fluctuate with different stages of placental development, and are altered in pregnancy pathologies. To test the hypotheses, the specific aims of the study were (1) to determine the gene expression profile of HTR8/SVneo trophoblast cells in response to different oxygen concentrations and (2) to determine the differential expression of hypoxia responsive genes in the placenta of early and late first trimester, term uncomplicated and term complicated pregnancy. This study has confirmed that HIFs are translocated to the nucleus of trophoblasts under replicated physiologically low oxygen conditions *in vitro* and possibly in the first trimester placenta *in situ.* This study has also demonstrated that the expression of hypoxia responsive genes in trophoblasts is increased in low oxygen tension *in vitro*.

4.2 Gene expression profiling of HTR8/SVneo trophoblast cells in response to different oxygen concentrations

HTR8/SVneo trophoblast cells were cultured in 1% and 5% oxygen atmospheres to mimic the placental oxygen environment of early first trimester and late first trimester, respectively. The localisation of active HIF-1α and HIF-2α was observed in the nucleus of HTR8/SVneo trophoblasts under 1% oxygen. Microarray analysis identified differential expression of 290 genes when comparing 1% and 5% oxygen environment in HTR8/SVneo trophoblast cells. HIF-1α was identified as a top upstream regulator of 41 differentially expressed genes, using Ingenuity Pathway Analysis. From these 41 genes, the expression

of *IGFBP3, IGFBP5, MMP1, VEGFA, P4HA1, P4HA2* and *ANGPTL4* was validated using qPCR. All of these genes, except for *MMP1*, were predicted to have HIF-1α transcription factor binding sites. In a similar study by Koklanaris and colleagues, 299 genes were found to be expressed differentially in HTR8/SVneo trophoblasts in 1% (hypoxia) oxygen compared to 20% (standard culture conditions) using microarray [215]. They found 215 genes to be upregulated in 1% oxygen including many involved in differentiation, motility/migration, angiogenesis and apoptosis. Some of their significantly up-regulated genes of interest including *NDRG1*, *ANGPTL4 and BNIP3*, were also up-regulated in our study, although the extent of fold change was lower. The difference in the fold change in the current study was probably due to using 5% oxygen instead of 20% as normoxia. Our study found that only 8 genes are expressed differentially in 5% vs 20% oxygen, suggesting that the effect of using 20% instead of 5% is negligible. Studies measuring the oxygen tension of the intrauterine environment report that the early first trimester placenta develops in 1-2% oxygen and the onset of maternal blood flow increases the oxygen tension to around 5-8% [65, 151]. Therefore 5% is a more appropriate concentration for studies of placental normoxia [151]. Koklanaris and colleagues also confirmed the up-regulation of six hypoxia responsive genes including *IGFBP3* in 1% oxygen compared 20% [215]. Several studies have reported the involvement of *IGFBP3* [170], *MMP1* [177], *VEGFA* [179] and *ANGPTL4* [211] with trophoblast functions. However, whether these genes act differently under hypoxic and normoxic conditions remains largely unknown. Previously we have found that HTR8/SVneo trophoblasts are more invasive in 1% oxygen compared to 5% (Khoda *et al*. unpublished). Under 1% oxygen condition HIFs likely facilitate the expression of *IGFBP3, MMP1, VEGFA, P4HA1, P4HA2* and *ANGPTL4* and these genes could play a pivotal role in trophoblast invasion.

4.3 Expression of hypoxia responsive genes in the placenta of early and late first trimester, term uncomplicated and term complicated pregnancy

Nuclear localisation of active HIFs was observed in the trophoblast of first trimester placenta along with decreased expression of HIF-1α and increased expression of HIF-2α in late first trimester. Expression of HIFs during the different time points of pregnancy has been reported in a study, where, both HIF-1α and HIF-2α protein decreased with increasing gestational age and the localisation of HIF-1α and HIF-2α was observed in both the nucleus and cytoplasm of syncytiotrophoblast, villous cytotrophoblast and feto-placental vasculature of 7 weeks, 9.5 weeks and uncomplicated term placenta [90]. In the current study, gestational age was used as the criterion for early and late first trimester. Before 9-10 weeks there is apparently no blood flow to the intervillous space of placenta [55] and hence 9 weeks was excluded from the selection in an attempt to exclude samples where blood flow status was ambiguous.

We have also determined the expression of hypoxia responsive genes of interest in the different stages of first trimester placenta, term uncomplicated and term complicated placenta. Expression of *IGFBP3, IGFBP5, VEGFA* and *ANGPTL4* was increased in term placenta compared to first trimester. IGFBPs in part regulate the bioavailability of IGFs and play a role in controlling fetal and placental development throughout gestation [169, 216]. *VEGFA* (reviewed in [217]) and *ANGPTL4* [197] are involved in angiogenesis and thus required for placental development. Increased expression of the hypoxia responsive genes in term placenta, which is unlike the expression pattern of HTR8/SVneo trophoblasts. This was unexpected and needs further investigation. Expression of hypoxia responsive genes in the term placenta also varies depending on which part of the placenta has been used as sample. A microarray study showed differential expression pattern of some hypoxia responsive genes in nine different sites of uncomplicated term placenta, starting from the placental centre to the lateral border and the basal to the chorionic plate [218]. They confirmed that the expression of *VEGF* along with connective tissue growth factor (CTGF), the cytoskeleton proteins lamininA3 and α-tubulin, and the signal transduction protein Rad are up-regulated in the subchorionic lateral border compared to medial basal site. Their findings were also correlated with villous histology where the upregulation of hypoxia

responsive gene associated with higher villous maturation, syncytial knots and fibrin deposits observed in the subchorionic placental lateral border. In the current study, term placenta samples were full thickness blocks for the histology and just villous tissue for gene expression analyses sampled about 4cm from the cord insertion site

Expression of hypoxia responsive genes in complicated term placenta compared to uncomplicated term placenta was also determined. Insufficient trophoblast invasion leading to hypoxia later in pregnancy is associated with many pregnancy complications. Preeclampsia (PE) may develop later in pregnancy as a result of impaired trophoblast remodelling of the spiral arterioles due to placental hypoperfusion and villous hypoxia [219], and consequently may lead to intrauterine growth restriction (IUGR). A study conducted in both primary term trophoblast culture *in vitro* and villous trophoblast *in vivo* identified a set of hypoxia regulated genes using high density oligonucleotide microarray [220]. They showed many genes including *VEGF* and *NDRG1*, which were upregulated in the current study, have highest expression in trophoblast exposed to ≤1% oxygen compared to 20% *in vitro* and in placenta from pregnancy complicated by IUGR compared to healthy control. Another microarray study reported the similar gene expression pattern between first trimester placenta explants treated with 3% oxygen, high altitude placenta and preeclamptic placenta [213]. Recently, altered expression of *IGFBP3* has been shown in placenta complicated with PE [221], IUGR [222] and preterm birth (PTB) [223] compared to uncomplicated placenta. Expression of *VEGFA* has been shown to be reduced in the placenta complicated by PE, GH, small for gestational age (SGA) and PTB compared to uncomplicated placenta [224]. However, in the current study, no difference was observed in the expression of any hypoxia responsive genes in complicated placenta compared to uncomplicated. Although it is believed that preeclamptic placentas are hypoxic, the actual oxygen tension of the preeclamptic placenta has not been measured yet. Interestingly, Huppertz *et al.* have suggested an increase of oxygen tension, rather than decrease, in preeclamptic placenta resulting from higher blood flow velocity caused by failure of spiral artery transformation [214]. Alternatively, higher blood flow velocity could limit oxygen

and nutrient transport across the placenta to the fetal circulation. However, in our study similar expression of hypoxia responsive genes in term control and complicated placenta suggests that oxygen levels in term control and complicated placentas are similar.

4.4 Limitations of the study

The expression pattern of hypoxia responsive genes in first trimester placenta compared to term control placenta was different from that of the first trimester trophoblast cell line. HTR8/SVneo cells were established from human first trimester cytotrophoblast [150] and it has been used widely for investigating first trimester cytotrophoblast behaviour. Within the placenta, extravillous cytotrophoblast cells, originate from chorionic villi at the interface between the cell column and placental bed, and are able to invade maternal decidua. After invasion, these invasive extravillous cytotrophoblasts further transform to endovascular cytotrophoblast. This ability to differentiate is acquired under the influence of the surrounding maternal environment and by the expression of some transmembrane proteins such as cadherin superfamily member and integrin subunits. *In vitro*, cytotrophoblasts, isolated from placenta, undergo systematic differentiation resulting in heterogeneous phenotypes similar to those observed *in vivo* [58, 225, 226]. In the current study, different patterns of the expression of hypoxia responsive genes in HTR8/SVneo trophoblast cell line and in the placenta is probably due to these factors. In addition, the genes of interest were selected based on differential expression in HTR8/SVneo trophoblasts under 1% and 5% oxygen representing early and late first trimester. It is not possible to know whether placenta samples collected from first trimester pregnancy terminations were normal or not and whether the pregnancies, if continued, would have been complicated or uncomplicated. Therefore, use of a trophoblast cell line allowed us to study the effect of oxygen tension on gene expression under controlled conditions in trophoblasts but may not faithfully recapitulate the *in vivo* situation where the placenta is intact and exposed to a variety of maternal decidual signals.

4.5 Significance and Conclusion

From all the findings, it can be concluded that expression of HIFs and hypoxia responsive genes is different in different oxygen environments correlating with reported increased trophoblast functions in low oxygen tension. A number of pregnancy complications are associated with insufficient trophoblast invasion. Understanding the role of oxygen tension in regulating trophoblast functions in healthy and complicated placenta will help in our understanding of how these complications develop and assist in prevention and identification of new therapeutic targets for these common diseases. Some pregnancy complications are known to increase the risk of developing life threatening cardiovascular and other diseases later in life in both the mother and the baby. Hence this research will also improve our knowledge about the effect of oxygen in early development of the placenta.

4.6 Future work

Our study provides further molecular evidence that oxygen influences trophoblast activity likely through HIF mediated factors. However, very little is known about how all of the identified hypoxia responsive genes regulate trophoblast activity. Further work could focus on the posttranslational effect of these genes on trophoblast cells. Knockdown of genes of interest which are involved in trophoblast functions and also regulated by HIF-1α, followed by invasion assay could reveal if HIF-1α is a “master regulator” of trophoblast invasion. Completing quantification of the identified genes of interest at the protein level by western blot in HTR8/SVneo cultured in hypoxia and normoxia, and subsequently in placental samples, could also provide further evidence for their roles in placental development.

References

1. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thromb Res 2004; 114:397-407.
2. Knofler M. Critical growth factors and signalling pathways controlling human trophoblast invasion. Int J Dev Biol 2010; 54:269-280.
3. Benirschke K, Kaufmann P. Pathology of the human placenta. Springer Verlag; 2000.
4. Moore KL, Persaud TVN. The Developing Human: Clinically Oriented Embryology Philadelphia, Pennsylvania: W. B. Saunders Company; 1998.
5. Frost JM, Moore GE. The importance of imprinting in the human placenta. PLoS Genet 2010; 6:e1001015.
6. Hilder L ZZ, Parker M, Jahan S, Chambers GM 2014 Australia's mothers and babies 2012 Perinatal statistics series no. 30 Canberra AIHW
7. Li Z, McNally L, L H, EA S 2011 Australian Mothers and Babies 2009 Australian Institute of Health and Welfare's Perinatal Statistics Sydney
8. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. Lancet 2005; 365:785-799.
9. Cross J. The genetics of pre-eclampsia: a feto-placental or maternal problem? Clinical genetics 2003; 64:96-103.
10. Varvarigou AA. Intrauterine growth restriction as a potential risk factor for disease onset in adulthood. J Pediatr Endocrinol Metab 2010; 23:215-224.
11. Ong KK, Dunger DB. Perinatal growth failure: the road to obesity, insulin resistance and cardiovascular disease in adults. Best Pract Res Clin Endocrinol Metab 2002; 16:191-207.
12. McCormick MC. The contribution of low birth weight to infant mortality and childhood morbidity. N Engl J Med 1985; 312:82-90.
13. Norwitz ER. Defective implantation and placentation: laying the blueprint for pregnancy complications. Reproductive BioMedicine Online 2006; 13:591-599.
14. Burton GJ, Watson AL, Hempstock J, Skepper JN, Jauniaux E. Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. Journal of Clinical Endocrinology and Metabolism 2002; 87:2954-2959.
15. Cariappa R, Heath-Monnig E, Smith CH. Isoforms of Amino Acid Transporters in Placental Syncytiotrophoblast: Plasma Membrane Localization and Potential Role in Maternal/Fetal Transport. Placenta 2003; 24:713-726.
16. Marin JJG, Macias RIR, Serrano MA. The Hepatobiliary-like Excretory Function of the Placenta. A Review. Placenta 2003; 24:431-438.
17. Corbacho AM, Martínez de la Escalera G, Clapp C. Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. Journal of Endocrinology 2002; 173:219-238.
18. Dutta-Roy AK. Transport mechanisms for long-chain polyunsaturated fatty acids in the human placenta1, 2, 3. The American journal of clinical nutrition 2000; 71:315S-322S.
19. Heron M. Deaths: leading causes for 2010. Natl Vital Stat Rep 2013; 62:1-97.
20. Gluckman PD, Hanson MA. Living with the past: evolution, development, and patterns of disease. Science 2004; 305:1733-1736.
21. Barker DJ BA, Osmond C, Simmonds SJ. Fetal and placental size and risk of hypertension in adult life. BMJ 1990; 301:259-262.
22. Brown MA, Lindheimer MD, de Swiet M, Assche AV, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). Hypertension in pregnancy 2001; 20.
23. Cartwright JE, Fraser R, Leslie K, Wallace AE, James JL. Remodelling at the maternal-fetal interface: relevance to human pregnancy disorders. Reproduction 2010; 140:803-813.

24. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science's STKE 2005; 308:1592.
25. Ness RB, Roberts JM. Heterogeneous causes constituting the single syndrome of preeclampsia: a hypothesis and its implications. Am J Obstet Gynecol 1996; 175:1365-1370.
26. Witlin AG, Saade GR, Mattar F, Sibai BM. Predictors of neonatal outcome in women with severe preeclampsia or eclampsia between 24 and 33 weeks' gestation. Am J Obstet Gynecol 2000; 182:607-611.
27. Egbor M, Ansari T, Morris N, Green CJ, Sibbons PD. Morphometric placental villous and vascular abnormalities in early- and late-onset pre-eclampsia with and without fetal growth restriction. BJOG 2006; 113:580-589.
28. Ogge G, Chaiworapongsa T, Romero R, Hussein Y, Kusanovic JP, Yeo L, Kim CJ, Hassan SS. Placental lesions associated with maternal underperfusion are more frequent in early-onset than in late-onset preeclampsia. J Perinat Med 2011; 39:641-652.
29. Roberts JM, Gammill HS. Preeclampsia: recent insights. Hypertension 2005; 46:1243.
30. Akhilesh M, Mahalingam V, Nalliah S, Ali RM, Ganesalingam M, Haleagrahara N. Hypoxia-inducible factor-1alpha as a predictive marker in pre-eclampsia. Biomed Rep 2013; 1:257-258.
31. Rajakumar A, Doty K, Daftary A, Harger G, Conrad KP. Impaired oxygen-dependent reduction of HIF-1alpha and -2alpha proteins in pre-eclamptic placentae. Placenta 2003; 24:199-208.
32. Sibai BM. Prevention of preeclampsia: A big disappointment. Am J Obstet Gynecol 1998; 179:1275-1278.
33. Hauth JC, Ewell MG, Levine RJ, Esterlitz JR, Sibai B, Curet LB, Catalano PM, Morris CD. Pregnancy outcomes in healthy nulliparas who developed hypertension. Obstetrics & Gynecology 2000; 95:24-28.
34. Buchbinder A, Sibai BM, Caritis S, MacPherson C, Hauth J, Lindheimer MD, Klebanoff M, VanDorsten P, Landon M, Paul R, Miodovnik M, Meis P, et al. Adverse perinatal outcomes are significantly higher in severe gestational hypertension than in mild preeclampsia. Am J Obstet Gynecol 2002; 186:66-71.
35. Bernstein IM, Horbar JD, Badger GJ, Ohlsson A, Golan A. Morbidity and mortality among very-low-birth-weight neonates with intrauterine growth restriction. American journal of obstetrics and gynecology 2000; 182:198-206.
36. Mayhew T, Wijesekara J, Baker P, Ong S. Morphometric evidence that villous development and fetoplacental angiogenesis are compromised by intrauterine growth restriction but not by pre-eclampsia. Placenta 2004; 25:829-833.
37. Teasdale F, Jean-Jacques G. Intrauterine growth retardation: Morphometry of the microvillous membrane of the human placenta. Placenta 1988; 9:47-55.
38. Ortiz-Mantilla S, Choudhury N, Leevers H, Benasich AA. Understanding language and cognitive deficits in very low birth weight children. Dev Psychobiol 2008; 50:107-126.
39. Murphy VE, Smith R, Giles WB, Clifton VL. Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. Endocrine Reviews 2006; 27:141-169.
40. Barker DJ. Human growth and cardiovascular disease. Nestle Nutr Workshop Ser Pediatr Program 2008; 61:21-38.
41. Howson CP, Kinney MV, Lawn JE. Born Too Soon: The Global Action Report on Preterm Birth. In. Geneva: World Health Organization; 2012.
42. Rogers LK, Velten M. Maternal inflammation, growth retardation, and preterm birth: insights into adult cardiovascular disease. Life Sci 2011; 89:417-421.
43. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. The Lancet 2008; 371:75-84.
44. Goldenberg RL, Gravett MG, Iams J, Papageorghiou AT, Waller SA, Kramer M, Culhane J, Barros F, Conde-Agudelo A, Bhutta ZA, Knight HE, Villar J. The preterm birth syndrome: issues to consider in creating a classification system. Am J Obstet Gynecol 2012; 206:113-118.

45. DeFranco E, Teramo K, Muglia L. Genetic influences on preterm birth. Semin Reprod Med 2007; 25:40-51.
46. Plunkett J, Muglia LJ. Genetic contributions to preterm birth: implications from epidemiological and genetic association studies. Ann Med 2008; 40:167-195.
47. Cooperstock M, Campbell J. Excess males in preterm birth: interactions with gestational age, race, and multiple birth. Obstet Gynecol 1996; 88:189-193.
48. Brettell R, Yeh PS, Impey LWM. Examination of the association between male gender and preterm delivery. European Journal of Obstetrics & Gynecology and Reproductive Biology 2008; 141:123-126.
49. Menon R. Spontaneous preterm birth, a clinical dilemma: etiologic, pathophysiologic and genetic heterogeneities and racial disparity. Acta Obstet Gynecol Scand 2008; 87:590-600.
50. Faye-Petersen O. The placenta in preterm birth. Journal of clinical pathology 2008; 61:1261-1275.
51. Kim YM, Chaiworapongsa T, Gomez R, Bujold E, Yoon BH, Rotmensch S, Thaler HT, Romero R. Failure of physiologic transformation of the spiral arteries in the placental bed in preterm premature rupture of membranes. Am J Obstet Gynecol 2002; 187:1137-1142.
52. Boyd JD, Hamilton WJ. The Human placenta. 1970.
53. Kliman HJ. Trophoblast infiltration. Reproductive Medicine Review 1994; 3:137-157.
54. Genbacev O, Krtolica A, Kaelin W, Fisher SJ. Human Cytotrophoblast Expression of the von Hippel–Lindau Protein Is Downregulated during Uterine Invasion in Situ and Upregulated by Hypoxia in Vitro. Developmental Biology 2001; 233:526-536.
55. Burton GJ, Jauniaux E, Watson AL. Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited. Am J Obstet Gynecol 1999; 181:718-724.
56. Martin Jr CB. Uterine blood flow and placental circulation. Anesthesiology 1965; 26:447.
57. Bischof P, Meisser A, Campana A. Paracrine and autocrine regulators of trophoblast invasion--a review. Placenta 2000; 21 Suppl A:S55-60.
58. Lala PK, Hamilton GS. Growth factors, proteases and protease inhibitors in the maternal-fetal dialogue. Placenta 1996; 17:545-555.
59. Pringle KG, Kind KL, Sferruzzi-Perri AN, Thompson JG, Roberts CT. Beyond oxygen: complex regulation and activity of hypoxia inducible factors in pregnancy. Hum Reprod Update 2010; 16:415-431.
60. Pringle KG, Tadros MA, Callister RJ, Lumbers ER. The expression and localization of the human placental prorenin/renin-angiotensin system throughout pregnancy: Roles in trophoblast invasion and angiogenesis? Placenta 2011; 32:956-962.
61. Morrish DW, Dakour J, Li H. Functional regulation of human trophoblast differentiation. J Reprod Immunol 1998; 39:179-195.
62. Lala PK, Chakraborty C. Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury. Placenta 2003; 24:575-587.
63. Godar S, Horejsi V, Weidle UH, Binder BR, Hansmann C, Stockinger H. M6P/IGFII-receptor complexes urokinase receptor and plasminogen for activation of transforming growth factor-beta1. Eur J Immunol 1999; 29:1004-1013.
64. Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN, Burton GJ. Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. Am J Pathol 2000; 157:2111-2122.
65. Burton GJ, Caniggia I. Hypoxia: Implications for Implantation to Delivery—A Workshop Report. Placenta 2001; 22, Supplement A:S63-S65.
66. Rodesch F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet Gynecol 1992; 80:283-285.

67. Jauniaux E, Greenwold N, Hempstock J, Burton GJ. Comparison of ultrasonographic and Doppler mapping of the intervillous circulation in normal and abnormal early pregnancies. Fertility and sterility 2003; 79:100-106.
68. Jauniaux E, Hempstock J, Greenwold N, Burton GJ. Trophoblastic oxidative stress in relation to temporal and regional differences in maternal placental blood flow in normal and abnormal early pregnancies. Am J Pathol 2003; 162:115-125.
69. Hustin J, Jauniaux E, Schaaps JP. Histological study of the materno-embryonic interface in spontaneous abortion. Placenta 1990; 11:477-486.
70. Jauniaux E, Zaidi J, Jurkovic D, Campbell S, Hustin J. Comparison of colour Doppler features and pathological findings in complicated early pregnancy. Hum Reprod 1994; 9:2432-2437.
71. Watson AL, Palmer ME, Jauniaux E, Burton GJ. Variations in expression of copper/zinc superoxide dismutase in villous trophoblast of the human placenta with gestational age. Placenta 1997; 18:295-299.
72. Watson AL, Skepper JN, Jauniaux E, Burton GJ. Changes in concentration, localization and activity of catalase within the human placenta during early gestation. Placenta 1998; 19:27-34.
73. Burton GJ, Woods AW, Jauniaux E, Kingdom JCP. Rheological and Physiological Consequences of Conversion of the Maternal Spiral Arteries for Uteroplacental Blood Flow during Human Pregnancy. Placenta 2009; 30:473-482.
74. Chaddha V, Viero S, Huppertz B, Kingdom J. Developmental biology of the placenta and the origins of placental insufficiency. Semin Fetal Neonatal Med 2004; 9:357-369.
75. Viero S, Chaddha V, Alkazaleh F, Simchen MJ, Malik A, Kelly E, Windrim R, Kingdom JC. Prognostic value of placental ultrasound in pregnancies complicated by absent end-diastolic flow velocity in the umbilical arteries. Placenta 2004; 25:735-741.
76. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW. Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions. Exp Mol Med 2004; 36:1-12.
77. Dunwoodie SL. The role of hypoxia in development of the Mammalian embryo. Dev Cell 2009; 17:755-773.
78. Safran M, Kaelin WG, Jr. HIF hydroxylation and the mammalian oxygen-sensing pathway. J Clin Invest 2003; 111:779-783.
79. Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 1995; 270:1230-1237.
80. Chen L, Endler A, Shibasaki F. Hypoxia and angiogenesis: regulation of hypoxia-inducible factors via novel binding factors. Exp Mol Med 2009; 41:849-857.
81. Daikoku T, Matsumoto H, Gupta RA, Das SK, Gassmann M, DuBois RN, Dey SK. Expression of hypoxia-inducible factors in the peri-implantation mouse uterus is regulated in a cell-specific and ovarian steroid hormone-dependent manner. Evidence for differential function of HIFs during early pregnancy. J Biol Chem 2003; 278:7683-7691.
82. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 2000; 88:1474-1480.
83. Fryer BH, Simon MC. Hypoxia, HIF and the placenta. Cell Cycle 2006; 5:495-498.
84. Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. Science 1997; 277:1669-1672.
85. Ashur-Fabian O, Yerushalmi GM, Mazaki-Tovi S, Steinberg DM, Goldshtein I, Yackobovitch-Gavan M, Schiff E, Amariglio N, Rechavi G. Cell free expression of hif1alpha and p21 in maternal peripheral blood as a marker for preeclampsia and fetal growth restriction. PLoS One 2012; 7:e37273.
86. Cowden Dahl KD, Fryer BH, Mack FA, Compernolle V, Maltepe E, Adelman DM, Carmeliet P, Simon MC. Hypoxia-inducible factors 1alpha and 2alpha regulate trophoblast differentiation. Mol Cell Biol 2005; 25:10479-10491.

87. Choi JH, Lee HJ, Yang TH, Kim GJ. Effects of hypoxia inducible factors-1alpha on autophagy and invasion of trophoblasts. Clin Exp Reprod Med 2012; 39:73-80.
88. Patel J, Landers K, Mortimer RH, Richard K. Regulation of Hypoxia Inducible Factors (HIF) in Hypoxia and Normoxia During Placental Development. Placenta 2010; 31:951-957.
89. Ietta F, Wu Y, Winter J, Xu J, Wang J, Post M, Caniggia I. Dynamic HIF1A regulation during human placental development. Biol Reprod 2006; 75:112-121.
90. Rajakumar A, Conrad KP. Expression, ontogeny, and regulation of hypoxia-inducible transcription factors in the human placenta. Biol Reprod 2000; 63:559-569.
91. Rocha S. Gene regulation under low oxygen: holding your breath for transcription. Trends Biochem Sci 2007; 32:389-397.
92. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 2001; 292:464-468.
93. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, et al. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 2001; 292:468-472.
94. Mole DR, Pugh CW, Ratcliffe PJ, Maxwell PH. Regulation of the HIF pathway: enzymatic hydroxylation of a conserved prolyl residue in hypoxia-inducible factor alpha subunits governs capture by the pVHL E3 ubiquitin ligase complex. Adv Enzyme Regul 2002; 42:333-347.
95. Tanimoto K, Makino Y, Pereira T, Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. EMBO J 2000; 19:4298-4309.
96. Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ, Maxwell PH. Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem 2000; 275:25733-25741.
97. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999; 399:271-275.
98. Koivunen P, Hirsila M, Gunzler V, Kivirikko KI, Myllyharju J. Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases. J Biol Chem 2004; 279:9899-9904.
99. McNeill LA, Hewitson KS, Claridge TD, Seibel JF, Horsfall LE, Schofield CJ. Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the beta-carbon of asparagine-803. Biochem J 2002; 367:571-575.
100. Freedman SJ, Sun ZY, Kung AL, France DS, Wagner G, Eck MJ. Structural basis for negative regulation of hypoxia-inducible factor-1alpha by CITED2. Nat Struct Biol 2003; 10:504-512.
101. Withington SL, Scott AN, Saunders DN, Lopes Floro K, Preis JI, Michalicek J, Maclean K, Sparrow DB, Barbera JP, Dunwoodie SL. Loss of Cited2 affects trophoblast formation and vascularization of the mouse placenta. Dev Biol 2006; 294:67-82.
102. Rossignol F, Vache C, Clottes E. Natural antisense transcripts of hypoxia-inducible factor 1alpha are detected in different normal and tumour human tissues. Gene 2002; 299:135-140.
103. Uchida T, Rossignol F, Matthay MA, Mounier R, Couette S, Clottes E, Clerici C. Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha. J Biol Chem 2004; 279:14871-14878.
104. Dubinsky V, Poehlmann TG, Suman P, Gentile T, Markert UR, Gutierrez G. Role of regulatory and angiogenic cytokines in invasion of trophoblastic cells. Am J Reprod Immunol 2010; 63:193-199.

105. Genbacev O, DiFederico E, McMaster M, Fisher SJ. Invasive cytotrophoblast apoptosis in pre-eclampsia. Hum Reprod 1999; 14 Suppl 2:59-66.
106. Soares MJ, Chakraborty D, Renaud SJ, Kubota K, Bu P, Konno T, Rumi MA. Regulatory pathways controlling the endovascular invasive trophoblast cell lineage. J Reprod Dev 2012; 58:283-287.
107. Adelman DM, Gertsenstein M, Nagy A, Simon MC, Maltepe E. Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes Dev 2000; 14:3191-3203.
108. Maltepe E, Krampitz GW, Okazaki KM, Red-Horse K, Mak W, Simon MC, Fisher SJ. Hypoxia-inducible factor-dependent histone deacetylase activity determines stem cell fate in the placenta. Development 2005; 132:3393-3403.
109. McKinnon T, Chakraborty C, Gleeson LM, Chidiac P, Lala PK. Stimulation of human extravillous trophoblast migration by IGF-II is mediated by IGF type 2 receptor involving inhibitory G protein(s) and phosphorylation of MAPK. J Clin Endocrinol Metab 2001; 86:3665-3674.
110. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, Semenza GL. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res 1999; 59:3915-3918.
111. Trollmann R, Klingmuller K, Schild RL, Rascher W, Dotsch J. Differential gene expression of somatotrophic and growth factors in response to in vivo hypoxia in human placenta. Am J Obstet Gynecol 2007; 197:601 e601-606.
112. Pringle KG, Kind KL, Thompson JG, Roberts CT. Complex Interactions Between Hypoxia Inducible Factors, Insulin-Like Growth Factor-II and Oxygen in Early Murine Trophoblasts. Placenta 2007; 28:1147-1157.
113. Bischof P. Endocrine, paracrine and autocrine regulation of trophoblastic metalloproteinases. Early Pregnancy 2001; 5:30-31.
114. Caniggia I, Mostachfi H, Winter J, Gassmann M, Lye SJ, Kuliszewski M, Post M. Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3). J Clin Invest 2000; 105:577-587.
115. Caniggia I, Winter J, Lye SJ, Post M. Oxygen and placental development during the first trimester: implications for the pathophysiology of pre-eclampsia. Placenta 2000; 21 Suppl A:S25-30.
116. Qian D, Lin HY, Wang HM, Zhang X, Liu DL, Li QL, Zhu C. Normoxic induction of the hypoxic-inducible factor-1 alpha by interleukin-1 beta involves the extracellular signal-regulated kinase 1/2 pathway in normal human cytotrophoblast cells. Biol Reprod 2004; 70:1822-1827.
117. Multhaupt HA, Mazar A, Cines DB, Warhol MJ, McCrae KR. Expression of urokinase receptors by human trophoblast. A histochemical and ultrastructural analysis. Lab Invest 1994; 71:392-400.
118. Kjoller L, Kanse SM, Kirkegaard T, Rodenburg KW, Ronne E, Goodman SL, Preissner KT, Ossowski L, Andreasen PA. Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. Exp Cell Res 1997; 232:420-429.
119. Graham CH, Fitzpatrick TE, McCrae KR. Hypoxia stimulates urokinase receptor expression through a heme protein-dependent pathway. Blood 1998; 91:3300-3307.
120. Lash GE, Otun HA, Innes BA, Bulmer JN, Searle RF, Robson SC. Low oxygen concentrations inhibit trophoblast cell invasion from early gestation placental explants via alterations in levels of the urokinase plasminogen activator system. Biol Reprod 2006; 74:403-409.
121. Pallotto EK, Kilbride HW. Perinatal outcome and later implications of intrauterine growth restriction. Clin Obstet Gynecol 2006; 49:257-269.
122. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP, et al. Excess placental soluble fms-like tyrosine

kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 2003; 111:649-658.
123. Rajakumar A, Whitelock KA, Weissfeld LA, Daftary AR, Markovic N, Conrad KP. Selective overexpression of the hypoxia-inducible transcription factor, HIF-2alpha, in placentas from women with preeclampsia. Biol Reprod 2001; 64:499-506.
124. Rajakumar A, Brandon HM, Daftary A, Ness R, Conrad KP. Evidence for the functional activity of hypoxia-inducible transcription factors overexpressed in preeclamptic placentae. Placenta 2004; 25:763-769.
125. Rajakumar A, Michael HM, Daftary A, Jeyabalan A, Gilmour C, Conrad KP. Proteasomal Activity in Placentas from Women with Preeclampsia and Intrauterine Growth Restriction: Implications for Expression of HIF-α Proteins. Placenta 2008; 29:290-299.
126. Helske S, Vuorela P, Carpen O, Hornig C, Weich H, Halmesmaki E. Expression of vascular endothelial growth factor receptors 1, 2 and 3 in placentas from normal and complicated pregnancies. Mol Hum Reprod 2001; 7:205-210.
127. Nishi H, Nakada T, Hokamura M, Osakabe Y, Itokazu O, Huang LE, Isaka K. Hypoxia-inducible factor-1 transactivates transforming growth factor-beta3 in trophoblast. Endocrinology 2004; 145:4113-4118.
128. Wang A, Rana S, Karumanchi SA. Preeclampsia: the role of angiogenic factors in its pathogenesis. Physiology (Bethesda) 2009; 24:147-158.
129. Weisz L, Oren M, Rotter V. Transcription regulation by mutant p53. Oncogene 2007; 26:2202-2211.
130. Levy R, Smith SD, Yusuf K, Huettner PC, Kraus FT, Sadovsky Y, Nelson DM. Trophoblast apoptosis from pregnancies complicated by fetal growth restriction is associated with enhanced p53 expression. Am J Obstet Gynecol 2002; 186:1056-1061.
131. Lash GE, Hornbuckle J, Brunt A, Kirkley M, Searle RF, Robson SC, Bulmer JN. Effect of Low Oxygen Concentrations on Trophoblast-Like Cell Line Invasion. Placenta 2007; 28:390-398.
132. Genbacev O, Joslin R, Damsky CH, Polliotti BM, Fisher SJ. Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. The Journal of Clinical Investigation 1996; 97:540-550.
133. Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D. Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. Hum Reprod Update 2007; 13:121-141.
134. Pollheimer J, Knofler M. Signalling pathways regulating the invasive differentiation of human trophoblasts: a review. Placenta 2005; 26 Suppl A:S21-30.
135. Bauer S, Pollheimer J, Hartmann J, Husslein P, Aplin JD, Knofler M. Tumor necrosis factor-alpha inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures. J Clin Endocrinol Metab 2004; 89:812-822.
136. Lala PK, Graham CH. Mechanisms of trophoblast invasiveness and their control: the role of proteases and protease inhibitors. Cancer Metastasis Rev 1990; 9:369-379.
137. Clark DE, Smith SK, He Y, Day KA, Licence DR, Corps AN, Lammoglia R, Charnock-Jones DS. A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. Biol Reprod 1998; 59:1540-1548.
138. Araki-Taguchi M, Nomura S, Ino K, Sumigama S, Yamamoto E, Kotani-Ito T, Hayakawa H, Kajiyama H, Shibata K, Itakura A, Kikkawa F. Angiotensin II mimics the hypoxic effect on regulating trophoblast proliferation and differentiation in human placental explant cultures. Life Sci 2008; 82:59-67.
139. Fafet P, Rebouissou C, Maudelonde T, Vignais ML. Opposite effects of transforming growth factor-beta activation and rho-associated kinase inhibition on human trophoblast migration in a reconstituted placental-endometrial coculture system. Endocrinology 2008; 149:4475-4485.

140. Karmakar S, Das C. Regulation of trophoblast invasion by IL-1beta and TGF-beta1. Am J Reprod Immunol 2002; 48:210-219.
141. Conrad KP, Benyo DF. Placental Cytokines and the Pathogenesis of Preeclampsia. American Journal of Reproductive Immunology 1997; 37:240-249.
142. Khong T, Robertson W. Spiral Artery Disease. New York: W. W. Norton & Company; 1992.
143. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995; 92:5510-5514.
144. Denley A, Cosgrove LJ, Booker GW, Wallace JC, Forbes BE. Molecular interactions of the IGF system. Cytokine and Growth Factor Reviews 2005; 16:421-439.
145. Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA, Detmar M. Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. Am J Pathol 1996; 149:293-305.
146. Richard DE, Berra E, Pouysségur J. Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1α in vascular smooth muscle cells. Journal of Biological Chemistry 2000; 275:26765-26771.
147. McMahon S, Charbonneau M, Grandmont S, Richard DE, Dubois CM. Transforming growth factor beta1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression. J Biol Chem 2006; 281:24171-24181.
148. Heidbreder M, Qadri F, Jöhren O, Dendorfer A, Depping R, Fröhlich F, Wagner KF, Dominiak P. Non-hypoxic induction of HIF-3α by 2-deoxy-d-glucose and insulin. Biochemical and Biophysical Research Communications 2007; 352:437-443.
149. Onogi A, Naruse K, Sado T, Tsunemi T, Shigetomi H, Noguchi T, Yamada Y, Akasaki M, Oi H, Kobayashi H. Hypoxia inhibits invasion of extravillous trophoblast cells through reduction of matrix metalloproteinase (MMP)-2 activation in the early first trimester of human pregnancy. Placenta 2011; 32:665-670.
150. Graham CH, Hawley TS, Hawley RC, MacDougall JR, Kerbel RS, Khoo N, Lala PK. Establishment and Characterization of First Trimester Human Trophoblast Cells with Extended Lifespan. Experimental Cell Research 1993; 206:204-211.
151. Jauniaux E, Watson AL, Hempstock J, Bao Y-P, Skepper JN, Burton GJ. Onset of Maternal Arterial Blood Flow and Placental Oxidative Stress: A Possible Factor in Human Early Pregnancy Failure. The American Journal of Pathology 2000; 157:2111-2122.
152. Du P, Kibbe WA, Lin SM. lumi: a pipeline for processing Illumina microarray. Bioinformatics 2008; 24:1547-1548.
153. Lin SM, Du P, Huber W, Kibbe WA. Model-based variance-stabilizing transformation for Illumina microarray data. Nucleic Acids Res 2008; 36:e11.
154. Mi H, Muruganujan A, Casagrande JT, Thomas PD. Large-scale gene function analysis with the PANTHER classification system. Nat. Protocols 2013; 8:1551-1566.
155. Buckberry S, Bianco-Miotto T, Hiendleder S, Roberts CT. Quantitative allele-specific expression and DNA methylation analysis of H19, IGF2 and IGF2R in the human placenta across gestation reveals H19 imprinting plasticity. PLoS One 2012; 7:e51210.
156. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome biology 2007; 8:R19.
157. Han VK, Bassett N, Walton J, Challis JR. The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: evidence for IGF-IGFBP interactions at the feto-maternal interface. The Journal of Clinical Endocrinology & Metabolism 1996; 81:2680-2693.

158. White CA, Dimitriadis E, Sharkey AM, Salamonsen LA. Interleukin-11 inhibits expression of insulin-like growth factor binding protein-5 mRNA in decidualizing human endometrial stromal cells. Mol Hum Reprod 2005; 11:649-658.
159. Lee BP, Rushlow WJ, Chakraborty C, Lala PK. Differential gene expression in premalignant human trophoblast: role of IGFBP-5. Int J Cancer 2001; 94:674-684.
160. Jin S, Li SW, Long J, Li L, Tan ZJ. [The role of progesterone in human early pregnancy is mediated by insulin-like growth factors binding protein1-3]. Sichuan Da Xue Xue Bao Yi Xue Ban 2006; 37:399-403.
161. Han JY, Kim YS, Cho GJ, Roh GS, Kim HJ, Choi WJ, Paik WY, Rho GJ, Kang SS, Choi WS. Altered gene expression of caspase-10, death receptor-3 and IGFBP-3 in preeclamptic placentas. Mol Cells 2006; 22:168-174.
162. Suman P, Gupta SK. Comparative analysis of the invasion-associated genes expression pattern in first trimester trophoblastic (HTR-8/SVneo) and JEG-3 choriocarcinoma cells. Placenta 2012; 33:874-877.
163. Renaud SJ, Kubota K, Rumi MA, Soares MJ. The FOS Transcription Factor Family Differentially Controls Trophoblast Migration and Invasion. J Biol Chem 2013.
164. Matjila M, Millar R, van der Spuy Z, Katz A. The differential expression of Kiss1, MMP9 and angiogenic regulators across the feto-maternal interface of healthy human pregnancies: implications for trophoblast invasion and vessel development. PLoS One 2013; 8:e63574.
165. Bulmer JN, Innes BA, Levey J, Robson SC, Lash GE. The role of vascular smooth muscle cell apoptosis and migration during uterine spiral artery remodeling in normal human pregnancy. FASEB J 2012; 26:2975-2985.
166. Gilkes DM, Bajpai S, Chaturvedi P, Wirtz D, Semenza GL. Hypoxia-inducible factor 1 (HIF-1) promotes extracellular matrix remodeling under hypoxic conditions by inducing P4HA1, P4HA2, and PLOD2 expression in fibroblasts. J Biol Chem 2013; 288:10819-10829.
167. Gilkes DM, Chaturvedi P, Bajpai S, Wong CC, Wei H, Pitcairn S, Hubbi ME, Wirtz D, Semenza GL. Collagen prolyl hydroxylases are essential for breast cancer metastasis. Cancer Res 2013; 73:3285-3296.
168. Koklanaris N, Nwachukwu JC, Huang SJ, Guller S, Karpisheva K, Garabedian M, Lee MJ. First-trimester trophoblast cell model gene response to hypoxia. Am J Obstet Gynecol 2006; 194:687-693.
169. Giudice L. Growth factors and growth modulators in human uterine endometrium: their potential relevance to reproductive medicine. Fertility and sterility 1994; 61:1.
170. Han V, Bassett N, Walton J, Challis J. The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: evidence for IGF-IGFBP interactions at the feto-maternal interface. Journal of Clinical Endocrinology & Metabolism 1996; 81:2680-2693.
171. Liao M, Tong P, Zhao J, Zhang Y, Li Z, Wang J, Feng X, Hu M, Pan Y. Prognostic value of matrix metalloproteinase-1/ proteinase-activated receptor-1 signaling axis in hepatocellular carcinoma. Pathol Oncol Res 2012; 18:397-403.
172. Ma H, Cai H, Zhang Y, Wu J, Liu X, Zuo J, Jiang W, Ji G, Zhang Y, Liu C, Zhu W, Yu L. Membrane palmitoylated protein 3 promotes hepatocellular carcinoma cell migration and invasion via up-regulating matrix metalloproteinase 1. Cancer Lett 2014; 344:74-81.
173. Inoue H, Mimori K, Shiraishi T, Kataoka A, Sadanaga N, Ueo H, Barnard GF, Mori M. Expression of tissue inhibitor of matrix metalloproteinase-1 in human breast carcinoma. Oncol Rep 2000; 7:871-874.
174. Pulukuri SM, Rao JS. Matrix metalloproteinase-1 promotes prostate tumor growth and metastasis. Int J Oncol 2008; 32:757-765.
175. Murray GI, Duncan ME, O'Neil P, Melvin WT, Fothergill JE. Matrix metalloproteinase–1 is associated with poor prognosis in colorectal cancer. Nature medicine 1996; 2:461-462.

176. Ito T, Ito M, Shiozawa J, Naito S, Kanematsu T, Sekine I. Expression of the MMP-1 in human pancreatic carcinoma: relationship with prognostic factor. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc 1999; 12:669.
177. Renaud SJ, Kubota K, Rumi MA, Soares MJ. The FOS transcription factor family differentially controls trophoblast migration and invasion. J Biol Chem 2014; 289:5025-5039.
178. Jiang Y, Zhu Y, Shi Y, He Y, Kuang Z, Sun Z, Wang J. Downregulation of SPARC expression inhibits the invasion of human trophoblast cells in vitro. PLoS One 2013; 8:e69079.
179. Charnock-Jones DS, Kaufmann P, Mayhew TM. Aspects of Human Fetoplacental Vasculogenesis and Angiogenesis. I. Molecular Regulation. Placenta 2004; 25:103-113.
180. Schiessl B, Innes BA, Bulmer JN, Otun HA, Chadwick TJ, Robson SC, Lash GE. Localization of angiogenic growth factors and their receptors in the human placental bed throughout normal human pregnancy. Placenta 2009; 30:79-87.
181. Lash GE, Cartwright JE, Whitley GSJ, Trew AJ, Baker PN. The Effects of Angiogenic Growth Factors on Extravillous Trophoblast Invasion and Motility. Placenta 1999; 20:661-667.
182. Semenza G. Signal transduction to hypoxia-inducible factor 1. Biochem Pharmacol 2002; 64:993-998.
183. Taylor CM, Stevens H, Anthony FW, Wheeler T. Influence of hypoxia on vascular endothelial growth factor and chorionic gonadotrophin production in the trophoblast-derived cell lines: JEG, JAr and BeWo. Placenta 1997; 18:451-458.
184. Shore VH, Wang TH, Wang CL, Torry RJ, Caudle MR, Torry DS. Vascular endothelial growth factor, placenta growth factor and their receptors in isolated human trophoblast. Placenta 1997; 18:657-665.
185. Lash GE, Taylor CM, Trew AJ, Cooper S, Anthony FW, Wheeler T, Baker PN. Vascular Endothelial Growth Factor and Placental Growth Factor Release in Cultured Trophoblast Cells Under Different Oxygen Tensions. Growth Factors 2003; 20:189-196.
186. Mazure NM, Chen EY, Laderoute KR, Giaccia AJ. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood 1997; 90:3322-3331.
187. Myllyharju J. Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. Matrix Biol 2003; 22:15-24.
188. Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 2004; 20:33-43.
189. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci 2010; 123:4195-4200.
190. Hynes RO. The extracellular matrix: not just pretty fibrils. Science 2009; 326:1216-1219.
191. Hofbauer KH, Gess B, Lohaus C, Meyer HE, Katschinski D, Kurtz A. Oxygen tension regulates the expression of a group of procollagen hydroxylases. Eur J Biochem 2003; 270:4515-4522.
192. Myllyharju J, Schipani E. Extracellular matrix genes as hypoxia-inducible targets. Cell Tissue Res 2010; 339:19-29.
193. Chang J, Jackson S, Wardale J, Jones SW. Hypoxia modulates the phenotype of osteoblasts isolated from knee osteoarthritis patients leading to under-mineralised bone nodule formation. Arthritis Rheumatol 2014.
194. Hou M, Cui J, Liu J, Liu F, Jiang R, Liu K, Wang Y, Yin L, Liu W, Yu B. Angiopoietin-like 4 confers resistance to hypoxia/serum deprivation-induced apoptosis through PI3K/Akt and ERK1/2 signaling pathways in mesenchymal stem cells. PLoS One 2014; 9:e85808.
195. Inoue T, Kohro T, Tanaka T, Kanki Y, Li G, Poh HM, Mimura I, Kobayashi M, Taguchi A, Maejima T, Suehiro JI, Sugiyama A, et al. Cross-enhancement of ANGPTL4 transcription by HIF1 alpha and PPAR beta/delta is the result of the conformational proximity of two response elements. Genome Biol 2014; 15:R63.

196. Khong TL, Thairu N, Larsen H, Dawson PM, Kiriakidis S, Paleolog EM. Identification of the angiogenic gene signature induced by EGF and hypoxia in colorectal cancer. BMC Cancer 2013; 13:518.
197. Gealekman O, Burkart A, Chouinard M, Nicoloro SM, Straubhaar J, Corvera S. Enhanced angiogenesis in obesity and in response to PPARgamma activators through adipocyte VEGF and ANGPTL4 production. Am J Physiol Endocrinol Metab 2008; 295:E1056-1064.
198. Oike Y, Yasunaga K, Suda T. Angiopoietin-Related/Angiopoietin-Like Proteins Regulate Angiogenesis. International Journal of Hematology 2004; 80:21-28.
199. Li H, Ge C, Zhao F, Yan M, Hu C, Jia D, Tian H, Zhu M, Chen T, Jiang G, Xie H, Cui Y, et al. Hypoxia-inducible factor 1 alpha–activated angiopoietin-like protein 4 contributes to tumor metastasis via vascular cell adhesion molecule-1/integrin β1 signaling in human hepatocellular carcinoma. Hepatology 2011; 54:910-919.
200. Pijnenborg R, Anthony J, Davey DA, Rees A, Tiltman A, Vercruysse L, Van Assche A. Placental bed spiral arteries in the hypertensive disorders of pregnancy. BJOG: An International Journal of Obstetrics & Gynaecology 1991; 98:648-655.
201. Goldman-Wohl D, Yagel S. Regulation of trophoblast invasion: from normal implantation to pre-eclampsia. Molecular and Cellular Endocrinology 2002; 187:233-238.
202. Kim YM, Bujold E, Chaiworapongsa T, Gomez R, Yoon BH, Thaler HT, Rotmensch S, Romero R. Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes. American Journal of Obstetrics and Gynecology 2003; 189:1063-1069.
203. Khong TY, Liddell HS, Robertson WB. Defective haemochorial placentation as a cause of miscarriage: a preliminary study. Br J Obstet Gynaecol 1987; 94:649-655.
204. Bianco-Miotto T, Chiam K, Buchanan G, Jindal S, Day TK, Thomas M, Pickering MA, O'Loughlin MA, Ryan NK, Raymond WA, Horvath LG, Kench JG, et al. Global Levels of Specific Histone Modifications and an Epigenetic Gene Signature Predict Prostate Cancer Progression and Development. Cancer Epidemiology Biomarkers & Prevention 2010; 19:2611-2622.
205. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3:RESEARCH0034.
206. Lofstedt H-MLFE, Nilsson TNRNS. H. Pietras A. Vallon-Christersson J. Borg A. Gradin K. Poellinger L. Pahlman S. Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell 2006; 10:413-423.
207. Holmquist-Mengelbier L, Fredlund E, Lofstedt T, Noguera R, Navarro S, Nilsson H, Pietras A, Vallon-Christersson J, Borg A, Gradin K, Poellinger L, Pahlman S. Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell 2006; 10:413-423.
208. Charnock-Jones DS, Kaufmann P, Mayhew TM. Aspects of Human Fetoplacental Vasculogenesis and Angiogenesis. I. Molecular Regulation. Placenta; 25:103-113.
209. Schiessl B, Innes BA, Bulmer JN, Otun HA, Chadwick TJ, Robson SC, Lash GE. Localization of Angiogenic Growth Factors and Their Receptors in the Human Placental Bed Throughout Normal Human Pregnancy. Placenta; 30:79-87.
210. Harris LK. IFPA Gabor Than Award lecture: Transformation of the spiral arteries in human pregnancy: Key events in the remodelling timeline. Placenta; 32:S154-S158.
211. Basak S, Duttaroy AK. cis-9,trans-11 conjugated linoleic acid stimulates expression of angiopoietin like-4 in the placental extravillous trophoblast cells. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 2013; 1831:834-843.
212. Schödel J, Oikonomopoulos S, Ragoussis J, Pugh CW, Ratcliffe PJ, Mole DR. High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. 2011.

213. Soleymanlou N, Jurisica I, Nevo O, Ietta F, Zhang X, Zamudio S, Post M, Caniggia I. Molecular Evidence of Placental Hypoxia in Preeclampsia. The Journal of Clinical Endocrinology & Metabolism 2005; 90:4299-4308.
214. Huppertz B, Weiss G, Moser G. Trophoblast invasion and oxygenation of the placenta: measurements versus presumptions. Journal of Reproductive Immunology; 101:74-79.
215. Koklanaris N, Nwachukwu JC, Huang SJ, Guller S, Karpisheva K, Garabedian M, Lee M-J. First-trimester trophoblast cell model gene response to hypoxia. American Journal of Obstetrics and Gynecology 2006; 194:687-693.
216. Irwin JC, Suen LF, Martina NA, Mark SP, Giudice LC. Role of the IGF system in trophoblast invasion and pre-eclampsia. Hum Reprod 1999; 14 Suppl 2:90-96.
217. Andraweera PH, Dekker GA, Roberts CT. The vascular endothelial growth factor family in adverse pregnancy outcomes. Hum Reprod Update 2012.
218. Wyatt SM, Kraus FT, Roh CR, Elchalal U, Nelson DM, Sadovsky Y. The correlation between sampling site and gene expression in the term human placenta. Placenta; 26:372-379.
219. Levy R, Smith SD, Chandler K, Sadovsky Y, Nelson DM. Apoptosis in human cultured trophoblasts is enhanced by hypoxia and diminished by epidermal growth factor. 2000.
220. Roh CR, Budhraja V, Kim HS, Nelson DM, Sadovsky Y. Microarray-based identification of differentially expressed genes in hypoxic term human trophoblasts and in placental villi of pregnancies with growth restricted fetuses. Placenta; 26:319-328.
221. Dubova EA, Pavlov KA, Lyapin VM, Kulikova GV, Shchyogolev AI, Sukhikh GT. Expression of Insulin-Like Growth Factors in the Placenta in Preeclampsia. Bulletin of Experimental Biology and Medicine 2014; 157:103-107.
222. Börzsönyi B, Demendi C, Nagy Z, Tóth K, Csanád M, Pajor A, Rigó Jr J, Joó József G. Gene expression patterns of insulin-like growth factor 1, insulin-like growth factor 2 and insulin-like growth factor binding protein 3 in human placenta from pregnancies with intrauterine growth restriction. In: J Perinat Med, vol. 39; 2011: 701.
223. Demendi C, Börzsönyi B, Nagy ZB, jr JR, Pajor A, Joó JG. Gene expression patterns of insulin-like growth factor 1, 2 (IGF-1, IGF-2) and insulin-like growth factor binding protein 3 (IGFBP-3) in human placenta from preterm deliveries: influence of additional factors. European Journal of Obstetrics and Gynecology and Reproductive Biology 2012; 160:40-44.
224. Andraweera PH, Dekker GA, Laurence JA, Roberts CT. Placental expression of VEGF family mRNA in adverse pregnancy outcomes. Placenta 2012.
225. Damsky C, Fitzgerald M, Fisher S. Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. Journal of Clinical Investigation 1992; 89:210.
226. Aplin JD. Implantation, trophoblast differentiation and hæmochorial placentation: mechanistic evidence in vivo andin vitro. J Cell Sci 1991; 99:681-692.