Regulation of angiogenic growth factors (sFLT-1 and PLGF) by CRH family of peptides under altered oxygen tension in trophoblast and endothelial cells

By

Rekha Miraskar

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Medicine

University of Warwick, Department of Medicine
September 2018

1 Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented (including data generated and data analysis) was carried out by me unless otherwise stated in the text.

Rekha Miraskar

Table of Contents

2 Abstract

Preeclampsia, a pregnancy specific vascular disorder, is one of the leading causes of maternal and fetal morbidity and mortality worldwide. Abnormal placental angiogenesis resulting from altered levels of angiogenesis regulators, such as soluble fms-like tyrosine kinase (sFLT-1), vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) has been implicated to be central in the pathophysiology of preeclampsia. sFLT-1 and PLGF have been recently proposed as one of the potential biomarkers to aid in the clinical diagnosis and prediction of preeclampsia. However, the molecular mechanisms underlying placental dysregulation of angiogenic molecules leading to endothelial dysfunction in preeclampsia remains as an enigma. Along with sFLT-1, factors such as corticotrophin releasing hormone (CRH) and cortisol that are secreted by placenta are elevated in serum of preeclamptic women. Recent studies have illustrated a cardinal role of the CRH family of peptides in the regulation of VEGF in various cells. Accumulating evidence suggests a mechanistic association between CRH related peptides and regulators of angiogenesis. Thus, this project aims to investigate their interactions in trophoblast and endothelial cells and establish a potential link between their signaling pathways, using in vitro models under altered oxygen tension. This project will also focus effect of SFLT-1 rich media on fusogenic machinery and endocrine capacity of BEWO cells under altered oxygen tension.

Using various techniques (western blot, ECLIA, QRT PCR, ELISA and MSD human angiogenesis panel assay), my preliminary data from BEWO and HUVECs cellular models showed that CRH related peptides probably regulates sFLT-1 and PLGF protein secretion in HUVECs, HTR8/SVneo cells but not in BEWO cells. In HTR8 cells, CRH has no effect on sFLT-1 transcription under normal and low oxygen tension, but increases sFLT-1 release under low oxygen tension. Under altered oxygen tension, CRH increases VEGF mRNA more compared to PLGF mRNA. a trend towards an increase in PLGF release by CRH is seen. CRH regulates angiogenic factors in trophoblasts under altered oxygen tension. CRH possibly favors angiogenic state under low oxygen tension at the transcription level,

although CRH appears to increase sFLT-1/PLGF ratio at protein secretion level. Hypoxia increases CRHR1 and decreases CRHR2 transcription. CRH decreases transcription at CRHR1 and CRHR2 mRNA under high oxygen tension. CRH reduces hypoxia induced upregulation of CRHR1 but not CRHR2 mRNA.

CRH was unable reverse hypoxia mediated impaired fusogenic machinery and biochemical differentiation in BEWO cells and unable to restore the syncytialization. Hypoxia increases CRHR1 and CRHR2 expression in BEWO cells. CRHR2 more predominately being expressed than CRHR1 in hypoxia. CRHRs are differentially regulated in syncytialised BEWO cells under altered oxygen tension. CRH blocks upregulation of CRHR1 gene expression in differentiated BEWO cells under high oxygen tension. CRH blocks CRHR1 in undifferentiated BEWO cells under low oxygen tension. CRHR1 is down regulated in syncytialised BEWO cells when exposed to altered oxygen tension. CRH when added to differentiating cells under low oxygen tension has no effect on its receptors. Moreover, sFLT-1 rich media mediates fusogenic machinery and under low oxygen tension. CRH further impairs this and CRHR1 is down regulated which is reversed under low oxygen tension.

Preliminary data from BEWO and HUVECs cellular models showed that CRH related peptides probably regulate sFLT-1 and PLGF protein secretion in HUVECs and HTR8/SVneo cells, but not in BEWO cells; CRH's effect varies in altered oxygen conditions. CRH possibly favors angiogenic state under low oxygen tension at the transcription level, although CRH appears to increase sFLT-1/PLGF ratio at protein secretion level. CRH was unable reverse hypoxia mediated impaired fusogenic machinery and biochemical differentiation in BEWO cells and unable to restore the syncytialization. When added to differentiating cells under low oxygen tension, CRH has no effect on its receptors. Moreover, sFLT-1 rich media mediates fusogenic machinery under low oxygen tension. CRH further impairs this and CRHR1 is downregulated which is reversed under low oxygen tension. The next step in research should focus on assessing the regulation of SFLT-1 and PLGF at transcriptional, translational and secretion level under altered oxygen tension and confirm the involvement of CRHRs in regulation of angiogenic and anti angiogenic factors.

3 Abbreviations

° C;	Degree Celsius
‘;	Minutes
11 β-HSD2;	11 Beta Hydroxysteroid Dehydrogenase 2
ACTH;	Adreno Corticotropic Hormone
AKT;	Serine/threonine-specific protein kinase or protein kinase B
Ang II;	Angiotensin II
AT 1AA;	Angiotensin II type I receptor Agonistic Autoantibodies
BCL-2;	B Cell Lymphoma 2
BMI;	Body mass index
cAMP;	Cyclic Adenosine Mono Phosphate or 3',5'-cyclic Adenosine Mono Phosphate
CB;	Carbenoxolone
cGMP;	Cyclic Guanosine Mono Phosphate
CRH;	Corticotrophin Releasing Hormone
CRHR;	Corticotrophin Releasing Hormone Receptor
eNOS;	Endothelial Nitric Oxide Synthase
ET1;	Endothelin 1
FORSKOLIN;	Forskolin
FBS;	Fetal Bovine Serum
FLT- 1;	Fms-Like Tyrosine kinase 1
GR;	Glucocorticoid Receptor
H;	Hours
HELLP syndrome;	Hemolysis Elevated Liver enzymes and Low Platelets syndrome
HIF-1α;	Hypoxia Inducible Factor 1-alpha
HPA;	Hypothalamus Pituitary Adrenal axis
KDR;	Kinase insert Domain Receptor
MR;	Mineralocorticoid Receptor
mRNA;	Messenger Ribo Nucleic Acid
NO;	Nitric Oxide
NOS;	Nitric Oxide Synthase
PBS;	Phosphate Buffer Saline
PLGF;	Placental Growth Factor
sENG;	soluble Endoglin
sFLT-1;	soluble Fms-Like Tyrosine kinase 1
sGC;	soluble Guanylate Cyclase
STBM;	Syncytiotrophoblast microparticles
TBS;	Tris Buffer Saline
TGF -β 1;	Transforming growth factor beta 1
UCN2;	Urocortin 2
VEGF;	Vascular Endothelial Growth Factor
β HCG;	Beta Human Chorionic Gonadotrophin

4 Introduction

4.1 Trophoblast differentiation

Normal placental development and function have long been identified to be very critical not only for the fetal development and survival in utero and its later life after birth but also for the well being of the mother during pregnancy and postpartum. Placentation involves trophoblast invasion and transformation of the maternal intramyometrial portion of the spiral arterioles, leading to the development of a low- impedance uteroplacental circulation. During embryonic development, an oxygen tension gradient exists between the villous placenta and maternal decidua. Hypoxia stimulates proliferation of cytotrophoblast and inhibits invasion of trophoblasts(Genbacev, Joslin et al. 1996).

At around 12 weeks' gestation, the intervillous space switches from relative hypoxia to a state of high oxygen tension (PO2 65 mm Hg). The stem cell of the placenta is the progenitor cytotrophoblast cell which proliferate throughout gestation, and differentiate along two pathways to form villous cytotrophoblast. This eventually can become syncytiotrophoblasts (outer cellular layer) or extravillous cytotrophoblasts (inner cellular layer; EVT); the latter has a proliferative component, an invasive component and a migratory EVT. Invasive EVT that invades decidua is called interstitial EVT, whereas EVT that invades and remodels the spiral arteries is called endovascular EVT. Endovascular invasion involves replacement of vascular smooth muscle and endothelial cells and transforms the spiral arteries into wide uteroplacental arteries. The occurrence of anastomoses between the dilated spiral arteries and endometrial veins form maternal sinusoids, thus leading to the development of the uteroplacental circulation. Endothelial cell surface markers are essential for endovascular EVT invasion. This is further supported with the research work which showed that in preeclampsia, the expression of endothelial cell markers in EVTs were reduced(Aplin 1991, Genbacev, Joslin et al. 1996)

The turnover of villous cytotrophoblast involves cytotrophoblasts' proliferation, differentiation and fusion or syncytialization, with the overlying syncytiotrophoblast (STB), thus causing syncytiotrophoblast to be continuously supplied with compounds

derived from the fusing cytotrophoblasts. This is further balanced by a concomitant release of apoptotic material as syncytial knots from the STB to the maternal circulation. The fusion or syncytialization is critical step for ensuring production of hormones and nutrient exchanges between the mother's and fetal blood. And thus essential for the maintenance of pregnancy and for fetal growth. The initiation of fusion has been shown to be regulated by key factors including protein kinases, cytokines, hormones, transcription factors, membrane proteins and proteases. Dysregulation of one or more of factors of fusion negatively affects the placental barrier maintenance and integrity. Fusion is defective in pregnancy-related complications such as preeclampsia, intrauterine growth restriction or trisomy 21(Gauster, Moser et al. 2009).

Signaling machinery: Studies have shown that adenylyl cyclase (AC) activation leads to enhanced expression of fusogenic gene mRNA and hormone secretion such as human chorionic gonadotropin (hCG) from placenta. Forskolin induced a rapid phosphorylation of ERK1/2 and p38MAPK; which further required PKA–AKAP interactions and led to downstream phosphorylation of CREB-1/ATF-1 via ERK1/2-dependent but p38MAPK-independent mechanisms. Both p38MAPK and ERK1/2 were involved in hCG-secretion induced by forskolin, suggesting the existence of additional p38MAPK-dependent but CREB-1/ATF-1-independent pathways. Upregulation of the mRNA expression of syncytin-1 and -2 (by 3- and 10-fold, respectively) the transcription factors such as old astrocyte specifically induced substance (OASIS) and glial cells missing a (GCMa) (by 3- and 6-fold, respectively) and the syncytin-2 receptor, major facilitator superfamily domain containing 2 (MFSD2) (by 2-fold) and AKAP79 and AKAP250 (by 2.5- and 4-fold, respectively) was seen in forskolin-treated BEWO cells. The effect of Forskolin on all these genes were suppressed by p38MAPK inhibition, whereas only few genes were sensitive to inhibition of ERK1/2(Shi, Lei et al. 1993, Keryer, Alsat et al. 1998, Daoud, Amyot et al. 2005, Vaillancourt, Lanoix et al. 2009, Delidaki, Gu et al. 2011).

In general, the initiation of cell-cell fusion occurs with the recognition and adhesion between two potentially fusing cells. This is further followed by formation of fusogenic pore, mixing of the cell surface bilayers resulting in cytoplasmic continuity. Trophoblast fusion is also implied to be controlled by the regulation of cell surface antigen, CD98, expressed in cytotrophoblast, ADAM proteins particularly ADAM12, calcium influx, AKT

activity as well as exposure of phosphatidylserine to the outer membrane leaflet syncytin 1 and 2 are thought to interact with neutral amino acid receptors, ASCT1 or 2 that function as syncytin receptors. Caspase 8 activation, microtubule associated protein stathmin, transcription factor GCMa and reduction of protein tyrosine phosphatase activity implicating increased tyrosine kinase activity are thought to be involved in the fusion process(Pötgens, Drewlo et al. 2004).

4.1.1 The role of oxygen in placental development

The placenta undergoes proliferation in hypoxic environment. Oxygen is a key regulator of trophoblast differentiation, proliferation, and migration in the development and function of placenta. During the initial low oxygen tension phase, the EVTs undergo key changes including a change in the expression of integrin profile, become migratory, and invade the decidual interstitium (interstitial EVT) and maternal spiral arteries (endovascular EVT). Although placental villi are bathed in maternal blood, prior to 10 weeks' gestation maternal blood flow to the placenta is blocked by endovascular EVT plugs. Thus, early development of placenta and embryo occurs in hypoxic environment, with nutrients supplied by maternal plasma and maternal endometrial glands secretion(Pijnenborg, Bland et al. 1981, Jauniaux, Jurkovic et al. 1991, Jaffe and Woods 1993, Burton, Jauniaux et al. 1999). This is further confirmed by doppler studies which showed the absence of blood flow into the intervillous space (IVS) prior to 10 weeks' gestation in normal pregnancies, with *in vivo* measurements indicating pO2 <20 mmHg. Excess reactive oxygen species as a result of premature perfusion of this IVS during this first 10 weeks' gestation increases the risk of pregnancy loss(Jauniaux, Watson et al. 1999, Jauniaux, Watson et al. 2000). Subsequently, the endothelial cells are replaced by EVTs within the maternal spiral arteries, the vascular smooth cells undergo apoptosis, and the lumen diameter increases, creating low resistance, high capacitance vessels that increase uteroplacental perfusion(Rodesch, Simon et al. 1992, Kam, Gardner et al. 1999, Pijnenborg, Ball et al. 2006).

The role of O2 in regulating EVT invasion may on one hand, depend on the cell types studied, conditions of study (in vitro versus in vivo), and gestational age from which the trophoblast cells are obtained. On the other hand, while the behavior of trophoblasts (ie,

proliferation and invasion) are sensitive to oxygen tension, the effect of persistent placental hypoxia, in vivo, on EVT proliferation/invasion in the endometrium may differ from those occurring in in vitro conditions. The role of hypoxia in EVT behavior thus remains a bit unclear. Initial studies have demonstrated that hypoxia promotes proliferation of trophoblasts, whereas high oxygen (O2) tension induces differentiation of trophoblasts. This is further supported by studies which showed that, proliferation was increased in 2% O2 compared to 21% O2, while invasion was inhibited, in isolated cultured CTBs, thus suggesting low O2 conditions inhibit invasion of endovascular EVTs. In contrast to this, a few studies have also shown that a hypoxia may promote EVT invasion(Rodesch, Simon et al. 1992, Genbacev, Zhou et al. 1997).

After 11 weeks of pregnancy, maternal blood from the decidual spiral arterioles circulates through the intervillous space so as to give appropriate nutrition for the fetus for the remaining gestation. By the third trimester of pregnancy, as fetoplacental oxygen consumption increases, free-floating villi that are exposed to an ambient pO2 of 60 mm Hg gradually declines to about 40 mm Hg(Soothill, Nicolaides et al. 1986) . Importantly, from here on, the delivery of oxygen and nutrients to the fetus is primarily the job of the free floating villi, with their bi-layer covering of trophoblasts normally bathed in maternalblood. During the first trimester, maternalspiral arteries maldevelopment predisposes to placental dysfunction and sub-optimal pregnancy outcomes in the second half of pregnancy.

Low oxygen tensions limit the cultured trophoblasts differentiation from uncomplicated pregnancies. This is reflected by impaired formation of differentiated syncytiotrophoblasts and decreased human chorionic gonadotropin and human placental lactogen secretions compared to trophoblasts exposed to standard conditions(Kudo, Boyd et al. 2003). Forskolin treated BEWO cells showed reduced intercellular fusion when cultured under limited oxygen tension (2% oxygen)(Kudo, Boyd et al. 2003). Syncytin is a retroviral gene product expressed in villous trophoblasts that reflects trophoblast differentiation as this protein is partly responsible for fusion of cytotrophoblasts with syncytiotrophoblasts (Blond, Lavillette et al. 2000, Frendo, Olivier et al. 2003). Moreover, preeclamptic placental villi exhibit decreased syncytin expression

and in vitro low oxygen tension decreases syncytin gene expression. Furthermore, under hypoxic conditions, reduced expression of syncytin and its receptor compared to control conditions (20% oxygen) in BEWO was observed. Moreover, at high altitude in vivo, third trimester villi exposed to hypoxia exhibit enhanced cytotrophoblast proliferation and decreased syncytiotrophoblasts fusion. In summary, these studies show that hypoxia impedes villous syncytiotrophoblast differentiation and thereby, may contribute to the altered cytodifferentiation as seen preeclamptic villi(Ali 1997, Lee, Keith et al. 2001, Knerr, Beinder et al. 2002, Mayhew, Bowles et al. 2002, Kudo, Boyd et al. 2003).

HIF-1 is a known transcriptional factor activated by hypoxia in many tissues for cellular adaptation to hypoxic conditions, by reducing oxygen consumption and metabolic activity. HIF-1, composed of HIF-1α and HIF-1β, may form a heterodimer, and then binds to promoters of oxygen-responsive genes. HIF-1 alpha expression is higher in the first trimester villi and the expression of HIF 1 alpha reduces in villi as the products of conception are exposed to oxygen tensions with the maternalblood perfusing the intervillous space. HIF-1α via the regulation of genes such as p53, p21, and Bcl-2 , may also regulate cellular proliferation and apoptosis(Carmeliet, Dor et al. 1998, Majmundar, Wong et al. 2010). The downstream regulation of several genes including the glucose transporter glut 1 and TGF beta3, whose expression in the placenta is known to inhibit differentiation, is affected by HIF1 alpha. Placentas of high altitude pregnancies showed a decreased expression of the GLUT 1 glucose transporter on the trophoblast basal plasma membrane, thus limiting the glucose transfer to yield smaller birthweight in conditions such as preeclampsia (Luscher, Marini et al. 2017) . HIF-1 alpha and TGF beta3 expressions are upregulated in preeclamptic placenta, which may limit the trophoblast differentiation during the second half of pregnancy. Soluble vascular endothelial growth factor receptor-1 (soluble fms-like tyrosine kinase 1, sFLT-1), which binds VEGF and placental growth factor (PLGF), is one of the downstream target genes of HIF-1 alpha. Higher maternal sFLT-1 secretions potentially sequester a bulk of free serum VEGF and could thereby impair the regulatory action of the growth factor. Taken together, these observations suggest that higher HIF-1 alpha expression in placental villi exposed to hypoxic conditions, such as preeclamptic pregnancy may adversely alter multiple placental functions(Kendall and Thomas 1993, Caniggia, Mostachfi et al. 2000).

Multiple cytokines are released differentially into the maternalcirculation by placental villi from preeclamptic compared to normotensive pregnancies. Similarly, the secretion of these cytokines are altered in cultured trophoblasts exposed to hypoxia. For example, hypoxia reduces IL-10 secretion but hypoxia increases IL-6 and IL-8 secretion. Moreover, hypoxia induces higher secretions of TNF alpha, IL-1 alpha, and IL-1 beta from cultured villi than control levels. TNF alpha was one of the first cytokines identified to induce apoptosis in placenta in vitro, and enhanced levels of TNF alpha released in response to villous hypoxia may contribute to the enhanced apoptotic turnover of trophoblasts in preeclampsia as described below. Collectively, these studies suggest that the response of villous trophoblast to hypoxia yields a cytokine secretion profile that is pro-inflammatory(Benyo, Miles et al. 1997, Maynard, Min et al. 2003, Keelan and Mitchell 2005).

4.2 Angiogenesis

Sprouting new blood vessels from existing ones is called angiogenesis(Chen and Zheng 2014). In a healthy adult body, after injury or insult, angiogenesis occurs for wound healing to restore blood flow to tissues. In female eutherians, angiogenesis occurs normally during the menstrual or estrous cycle to transform the ovulated follicles into the corpus luteum for synthesis of progesterone and to rebuild the uterine endometrium receptive for the implanting embryos. Angiogenesis also occurs in various pathological conditions such as cancer and retinopathy (Carmeliet 2003). This involves steps such as proliferation, migration, and differentiation of endothelium within the preexisting blood vessels as they send out capillary sprouts to initiate the new tube-like structures formation, and secondary vasodilatation to enhance oxygen and nutrient uptake and circulation(Chen and Zheng 2014). This multi-step process commences with an increase in local and/or systemic angiogenic factors, followed by endothelial basement membrane breakdown to facilitate migration and proliferation of endothelium. The differentiation of endothelium leads to formation of new tube-like structures that stabilizes as mature vessels with the pericytes or smooth muscle cells recruitment(Carmeliet 2000). Aberrant angiogenesis majorly contributes to the pathogenesis of numerous vascular diseases that are caused by either excessive angiogenesis in tumors, and cavernous hemangioma and retinopathy, or defective angiogenesis in diabetes, atherosclerosis, restenosis and

hypertension(Carmeliet 2003).

In eutherians, shortly after the embryo implantation, its trophectoderm develops into the placenta. It supports the fetal growth, development, and survival. The placental formation, growth, and function are regulated and coordinated with precision to operate the bi-directional nutrients and respiratory gases (oxygen and carbon dioxide) exchange and to exhaust metabolic wastes between mother and fetus at the maximal efficiency, which is executed through the circulatory system at the maternal, fetal and placental unit. This is to provide the support needed for early life of a mammal in the womb (Cross, Werb et al. 1994, Reynolds and Redmer 2001).

Angiogenesis in the placenta takes similar steps as it occurs in any other organs; it also requires proliferation, migration, and differentiation of endothelial cells within the preexisting trophoblastic micro vessels(Kaufmann, Mayhew et al. 2004). However, unlike pathological angiogenesis, angiogenesis occurring in the placenta is a normal physiological process that must be tightly regulated during pregnancy. Deranged placental vasculature has been identified in multiple pregnancy complications in women as well as animals(Mayhew, Charnock-Jones et al. 2004, Redman and Sargent 2005, Reynolds, Caton et al. 2006), thus emphasizing the importance of placental angiogenesis during pregnancy. Further investigations for in-depth understanding of the genetic, epigenetic, molecular, physiological and pathological regulation of placental angiogenesis are warranted, which enables to use placental angiogenesis as a target for the diagnostic and therapeutic purpose for pregnancy complications.

4.2.1 **Placental vascular formation and development**

Carcinogenesis is the process of de novo vascular formation that occurs during embryogenesis which commences with the formation of the angioblasts, endothelial progenitor cells in the extraembryonic mesoderm allantois(Cross 2003). During pregnancy, The vasculature of placenta further expands and elaborates with the placental morphogenesis(Burton, Charnock-Jones et al. 2009). Angiogenesis occurs in both the maternaland fetal placental tissues in an extensive way, thus leading to the development of placenta as a highly vascularized organ during late gestation. For

example, the capillary network in a normal human placenta is estimated to be 550 km in length and 15 square meters in surface area(Burton and Jauniaux 1995) . Extensive placental neovascularization is associated with periodic increase in the blood flow in uterine and placental during gestation. during implantation and placentation, Blood flows to the maternal- feto-placental units are established, gradually increases throughout the gestation, essentially keeping pace with the rate of the growing fetus (Reynolds and Redmer 2001). studies in animals have clearly shown that uterine and placental vessels vasodilatation and angiogenesis are the two important mechanisms to increase placental (umbilical cord) blood flow during late gestation, which is essential for normal growth and survival of fetus and is also directly linked to the maternalfetal well-being during pregnancy and postpartum (Reynolds, Caton et al. 2006).

4.2.2 **Trophoblast regulation of placental angiogenesis**

Since trophoblast cells are in close contact with the endothelial cells in the placenta; trophoblast-derived factors are thought to have a key role in the regulation of vascular formation and morphogenesis of placenta. As a primary active site of angiogenesis, the placenta is one of the important sources of both pro- and anti-angiogenic factors. During the third trimester of both human and ovine pregnancy, the period of exponential maternal-fetal interface vascular growth, blood flow, and fetal weight, the maternal-fetal compartments of the placentas produce numerous angiogenic factors, including PLGF, VEGF, endocrine gland-derived-VEGF, FGF2, transforming growth factor-β1 (TGF-β1), angiopoietins, leptin, and Slit/Robo signaling cues. Placenta also synthesizes a large number of anti-angiogenic factors, i.e., soluble VEGFR1 (sFLT-1) and soluble TGF-β1 receptor endoglin, etc., that are important for the fine tuning of placental angiogenesis(Senger, Galli et al. 1983, Derynck, Jarrett et al. 1985, Gospodarowicz, Ferrara et al. 1987, Keck, Hauser et al. 1989, Leung, Cachianes et al. 1989, Maglione, Guerriero et al. 1991, Zhang, Proenca et al. 1994, Sato, Tozawa et al. 1995, LeCouter, Kowalski et al. 2001, Levine, Maynard et al. 2004).

4.2.3 **Angiogenesis: branching and non-branching**

During the course of 9 months, the human placenta develops into an organ of a highly vascular capillary networks. These are essential for effective materno-fetal exchange, but

also plays a key mechanistic role in the elaboration of the placental villous tree. Vasculogenesis and subsequent angiogenesis are therefore of pivotal importance in placental development, and it is imperative that they are appropriately regulated. Failure of appropriate regulation of placental angiogenesis results in impoverished development of the placenta, leading ultimately to poor obstetric outcome such as IUGR, PE(Burton, Charnock-Jones et al. 2009).

Vasculogenesis begins as early as the third week post-conception. Under the influence of factors such as VEGF secreted by the overlying trophoblast, Hemangioblastic cell cords differentiate *in situ* from mesenchymal cells in the villous cores. The cords through cell recruitment and proliferation and, elongate and connect with the vasculature of the developing fetus. By around 8 weeks of gestation, a feto-placental circulation starts. The capillaries elongation outstrips that of the containing villi, leading to looping of the vessels. Both capillary loops and new sprouts obtrusion results in the terminal villi formation. Therefore, Branching and non-branching angiogenesis play important roles in villous morphogenesis throughout pregnancy. The circulating levels of VEGFA and placental growth factor in maternal sera vary across normal pregnancy, and in complicated pregnancies. It is difficult to determine the impact of these changes on placental angiogenesis, because the relationship between growth factors levels in the maternal circulation and their effects on fetal vessels within the placenta remains unclear. Moreover, the trophoblast releases large amounts of soluble receptors influencing their bioavailability by binding to both growth factors. The endothelial cells in placenta are prone to oxidative stress leading to the activation of the apoptotic cascade(Leach and Leach 2002, Kaufmann, Mayhew et al. 2004, Tertemiz, Kayisli et al. 2005, Jirkovska, Janacek et al. 2008, Burton, Charnock-Jones et al. 2009).

Oxidative stress correlated with the onset of the maternal circulation, and with incomplete spiral arteries remodeling in pathological pregnancies, plays an important role in sculpting the villous tree. A major switch in the pattern of angiogenesis within the placenta during pregnancy has been postulated. While the branching angiogenesis, the formation of new vessels through sprouting, occurring from day 32 pc to week 24, the non-branching angiogenesis, the formation of capillary loops through elongation,

predominates thereafter until term(Kaufmann, Mayhew et al. 2004, Benirschke, Kaufmann et al. 2006). This postulated hypothesis is based on the assumption that the during the second half of pregnancy, terminal villi elaboration, is driven by the capillary loops obstruction from the surface of intermediate villi as described previously. Furthermore, an increase in maternal PLGF levels during mid gestation causing suppression of VEGF A activity inhibit capillary sprouting. This change in angiogenic profile is linked to the increase in concentrations of intraplacental oxygen and is associated with maternal circulation onset. This hypothesis is challenged by the three-dimensional reconstructions evidence, which regularly demonstrated mature placentas terminal villi containing blind-ending capillary sprouts(Jirkovska, Janacek et al. 2008). During the second half of gestation, when the terminal villi in the placenta are formed, the occurrence of increased capillary branching has been suggested from the data obtained from high altitude pregnancies.

During pregnancy, the growth factors bioavailability at the villous level is difficult to predict due to the secretion of soluble receptors. The mode and rate of angiogenesis is unlikely to be dictated by one factor alone, but rather by a complex series of signaling mechanisms. Two important contributors are oxygen and growth factors. Indeed, an analysis of villous vascularization quantitatively across the first to second trimester transition revealed no sudden changes in response to the rising oxygen levels. During pregnancy, although there may be changes in the pattern of angiogenesis in placenta, the hypothesis that there is a dichotomous sFLT-1 from branching to non-branching angiogenesis during mid-pregnancy in a clear cut manner no longer appears justifiable(Jauniaux, Burton et al. 1991).

4.2.4 **Mechanical factors in the regulation of placental angiogenesis**

In comparison with oxygen and growth factors, surprisingly, the role of mechanical forces in the regulation of placental angiogenesis in placenta has not received attention, despite the existence of evidence indicating that they are powerful inducers of endothelial cell motility and proliferation. Some evidence that may be vital in the placenta is postulated by the observation that until the beginning of the second trimester of pregnancy, by which time a fetal circulation through the organ has been established, the recruitment

of perivascular cells as precursor smooth muscle cells, and the transformation into arteries and veins, is not observed(Kaufmann, Mayhew et al. 2004, Shiu, Weiss et al. 2005).

Shear stress may be an essential factor in the larger vessels of stem and intermediate villi, but at the level of terminal villi, the flow rate is not likely to be adequate to generate significant forces. Given the fact that acute changes in direction must take place within the capillary plexus, cyclic strain is probably more vital at these sites. This may elucidate the dilated sinusoids location at the apices of capillary loops(Burton, Tham et al. 1992). The observation that the capillary sinusoids are capable of compression / expansion dependent upon the pressure differential between the maternal and fetal circulations suggests that the capillary walls have elastic properties(Karimu, Burton et al. 1994, Karimu and Burton 1994, Mayhew, Charnock Jones et al. 2004). The pressure differential is likely to increase during pregnancy as the fetal heart matures, and so one might predict a continually elevating distending force being applied. This will be challenged by the complement of intermediate fibres within the endothelial cells, and also by the extracellular matrix composition and the existence of encircling collagen fibres. The communication between local growth factors and mechanical forces in sculpting vasculosyncytial membranes requires further investigation, for these sites are of key significance to materno-fetal exchange.

4.2.5 **Dysregulation of angiogenesis in diseases**

Several pathological conditions occur either by excess, dysregulated angiogenesis such as rheumatoid arthritis, retinopathy, psoriasis and tumor diseases, myocardial and limb and ischemia(Mayhew, Charnock Jones et al. 2004).

4.2.6 **Dysregulation of placental angiogenesis in pathological pregnancies**

Placental insufficiency causes the baby to receive inadequate oxygen and nutrients from the mother, due to inadequate functioning of placenta. This results in fetal stress,

challenging the growth of the baby. Placental Vascular development is frequently disturbed in pregnancy complications(Mayhew, Charnock-Jones et al. 2004). Although the changes may be inflicted by several potential factors in the intrauterine environment, significant attention has been focused on oxygen; both placental hypoxia and hyperoxia. Hypoxia, due to the maternal arterial supply compromise, is believed to cause exaggerated branching of capillaries and villi. These are most likely due to subtle alterations in the VEGF: PLGF ratio, however no firm evidence exists to confirm this hypothesis. Enhanced branching may enhance placental efficiency by offering more arterio-venous circuits functioning in parallel, returning fetal blood soon after it has equilibrated with that in the intervillous space, to the umbilical cord. Nevertheless, exaggerated stimulation of the endothelial cells with hypoxia result in benign tumors termed chorioangiomas. In some forms of the tumor, development of large vessels occurs and can lead to detrimental arterio-venous shunts, whereas in others there is only disorganized endothelial hyperplasia. Chorioangiomas often occurs at high altitudes in excess of 4000 m, thus indicating that hypoxia plays an important role in their etiology(Reshetnikova, Burton et al. 1996).

Hyperoxia is thought to have the opposite effect, causing decreased capillary branching and terminal villi. It is argued that these changes will decrease oxygen extraction from the intervillous space, so fostering further hyperoxia. While this may be factual it is a rather circular argument, and the initial aggravating cause of the vascular maldevelopment of placenta is not clear(Kingdom, Kaufmann et al. 1997).

Hypoxia and hyperoxia are relative terms, and as reviewed earlier the maternalblood flow pattern through the placental lobule means that villi in various regions will experience different concentrations of oxygen. The terms thus have to be qualified according to the location and the stage of pregnancy that is being considered. Consistency of the prevailing concentration of oxygen may be a more important than the absolute value. It has been hypothesized that the inadequate conversion of the maternalspiral arteries in association with the large number of pregnancy complications enhances the risk of spontaneous vasoconstriction, and hence predisposes the placenta to ischemia–reperfusion-type injury. High levels of oxidative stress occur in the trophoblast and endothelial cells, occur When normal placental explants are exposed to

hypoxia–reoxygenation *in vitro*, thus mimicking the changes seen in preeclampsia. Placenta from uncomplicated pregnancies following labour and vaginal delivery has shown same distribution of oxidative stress, while it is absent from those delivered by caesarean section(Hung, Skepper et al. 2001, Cindrova-Davies, Yung et al. 2007).

Oxidative stress is a powerful inducer of apoptosis, and this has been observed following labor and also hypoxia–reoxygenation *in vitro* via activation of the executioner caspase-3 in villous endothelial and trophoblast cells. It has thus been speculated that oxidative stress, as a result of impaired spiral artery conversion, leads to arrest of angiogenesis in placenta or even deterioration of existing capillaries. Consequently, high-resistance waveforms are observed in the umbilical circulation, and the reduced diffusing capacity of the organ is associated fetal hypoxia and growth restriction(Kuzmina, Hubina-Vakulik et al. 2005).

4.2.7 **Oxygen as a regulator of placental angiogenesis**

VEGF, PLGF can be acutely regulated by the local oxygen concentration, and hence attention has recently been focused on oxygen as a key factor controlling placental development. VEGFA is modulated at the level of transcription and also through mRNA stability, with low- concentrations of oxygen inducing the expression in placental fibroblasts(Wheeler, Elcock et al. 1995, Burton, Charnock-Jones et al. 2009). The translation of protein is also acutely modulated by the local oxygen concentration. PLGF is regulated in a similar fashion but in an opposite direction, being inhibited at low oxygen concentrations(Ahmed, Dunk et al. 2000). It is now widely accepted that the during the first trimester of pregnancy, the development of human placenta takes place in the absence of a significant maternal blood flow, conditions that would be expected to support vasculogenesis and angiogenesis. In the absence of experimental manipulations, it is difficult to establish how critical this environment is, but there is indirect evidence that this may be important. First, enhanced numbers of hemangiogenic cords are observed in early placental villi from pregnancies complicated by maternalanemia, when the oxygen levels in the developing placenta might be expected to be even lower than normal. Second, comparisons between central and peripheral regions of the placenta villi suggest that hyperoxia has the opposite effect. Ultrasonography has shown that maternalcirculation to the placenta begins at the end of the first trimester in a

peripheral–central fashion. As a result, the oxygen tension will be higher in the periphery, which is further supported by increased oxidative stress concentration. These peripheral villi either contain degenerate capillaries or are avascular, coherent with VEGF support withdrawal secondary to a hyperoxic environment(Zhang, Proenca et al. 1994, Jauniaux, Hempstock et al. 2003). Similar changes are evident in missed miscarriage placentas that are retained *in utero*, where there is premature onset of the maternal blood flow. At the end of the first trimester, oxygen levels within the intervillous space of placenta normally increases upto threefold. Which gradually falls from 16 weeks, from ~60 to 40 mmHg at term, as oxygen consumption within placenta and fetus rise(Jauniaux, Watson et al. 2000). The latter figures were obtained at the time of cordocentesis by aspiration of intervillous blood through the chorionic plate; and while providing an essential guide to placental oxygenation, they however do not consider the regional variations in oxygen levels that may play a key role in regulating the morphogenesis of villi. Hence, when maternal blood from the spiral arteries is supplied into the placenta, it is directed to the center of a placental lobule, from which it then infiltrates to the periphery. Thus, creating an oxygen gradient across the lobule, but for practical and ethical reasons, this cannot be confirmed by direct analysis using current technologies. However, the analysis of expression and activity of antioxidant enzyme in villous tissues obtained from different sites within placental lobules support this hypothesis. It is also notable that dilated capillary sinusoids and vasculosyncytial membranes are predominant in the peripheral regions of placental lobule, where low oxygen concentrations are expected(Fox 1967, Critchley and Burton 1987).

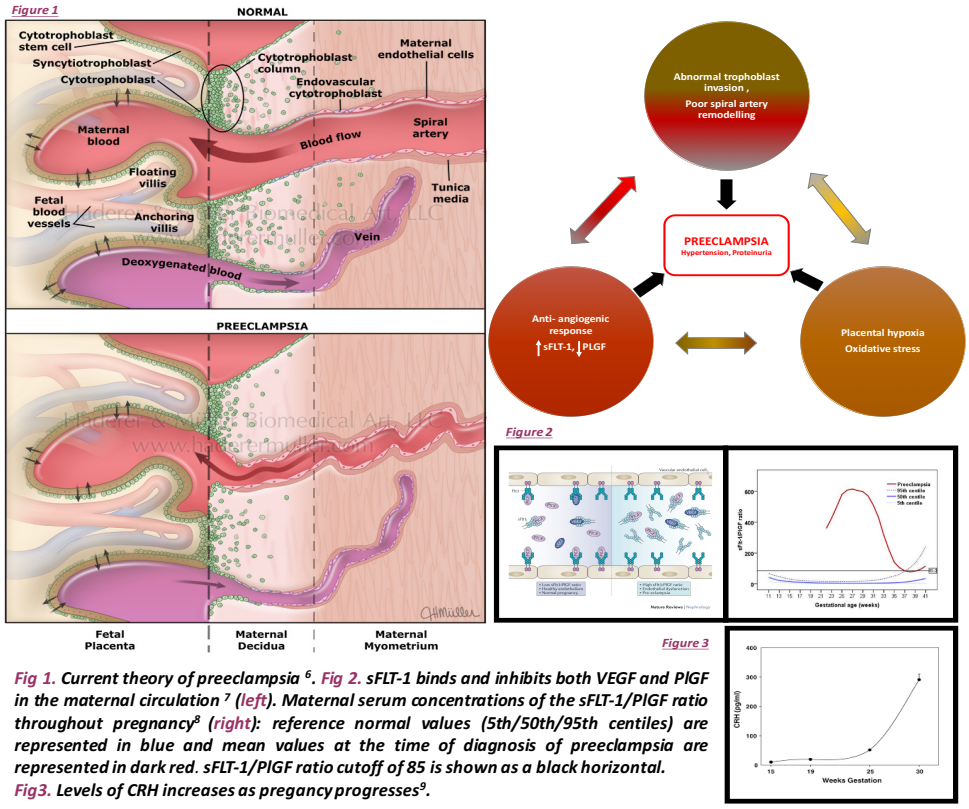


Fig 1. Current theory of preeclampsia [6]. Fig 2. sFLT-1 binds and inhibits both VEGF and PlGF in the maternal circulation [7] (left). Maternal serum concentrations of the sFLT-1/PlGF ratio throughout pregnancy[8] (right): reference normal values (5th/50th/95th centiles) are represented in blue and mean values at the time of diagnosis of preeclampsia are represented in dark red. sFLT-1/PlGF ratio cutoff of 85 is shown as a black horizontal.
Fig3. Levels of CRH increases as pregancy progresses[9].

Figure 1

Pathogenesis of preeclampsia

(http://www.haderermuller.com/portfolio-item/placenta-in-preeclampsia/; http://www.biochemsoctrans.org/content/37/6/1237.full; https://www.semanticscholar.org/paper/Angiogenesis-Related-Biomarkers-(sFLT-1%2FPLGF)-in-of-Herraiz-Simón/a600e04d87aa0e1affad0f698a943de707f39df4/figure/0; https://www.sciencedirect.com/science/article/abs/pii/S0196978105004705)

4.2.8 Angiogenic growth factors and their receptors:

4.2.8.1 The VEGF/VEGFR Family

VEGF-VEGFR system plays autocrine and paracrine role in the regulation of angiogenesis in placenta. The VEGF/VEGFR family consists of three membrane bound receptors: VEGFR-1 (also known as FLT-1), VEGFR-2 (also known as KDR in humans; Flk-1 in rodents) and VEGFR-3. The three VEGF receptors can interact with up to five ligands: VEGF (also known as VEGF-A), VEGF-B, VEGF-C, VEGF-D, and PLGF. A number of coreceptors, principally neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2), have also been identified that, when integrated into the receptor-ligand complex, further increases ligand mediated signaling (Ferrara, Gerber et al. 2003). These are all tyrosine kinases receptors. Following

ligand binding, the receptors dimerise before undergoing auto phosphorylation, leading to activation of the receptor.

Tyrosine kinase receptors play key roles in modulating the cellular metabolism, cell proliferation, migration, differentiation, apoptosis and cell cycle. The VEGF family plays a crucial role not only in vasculogenesis, angiogenesis and lymphangiogenesis, but also in non-vascular roles, such as in ovarian follicle formation and menstruation, fetal lung development, renal glomerular development and function, bone formation and haematopoiesis, neurogenesis and motor neuron survival(Ferrara, Gerber et al. 2003, Qiu, Hoareau-Aveilla et al. 2009). Both VEGFR1 and VEGFR2 is structurally distinct from VEGFR3. VEGF-C and VEGF-D binds to VEGFR3. VEGFR3 appears essential during lymphangiogenesis as well as embryogenesis for the cardiovascular system development, as evident with dying VEGFR-3 null mice at embryonic day E9.5 with cardiovascular failure. VEGFR-1 and VEGFR-2, in contrast to VEGFR-3, are structurally homologous both consisting of an extracellular domain containing seven immunoglobulin (Ig)-like domains, a transmembrane domain and an intracellular tyrosine kinase domain(Takahashi 2011).

Both soluble and membrane bound VEGFR-1 bind VEGF, VEGF-B, and PLGF, whereas VEGFR-2 binds VEGF, as well as processed forms of VEGF-C and VEGF-D. Although both soluble and membrane bound VEGFR-1 are structurally homologous and can bind to VEGF, their roles are suggested to be significantly different. VEGFR-1 primarily has been thought to act as 'VEGF trap', thus sequestering VEGF and deviating this ligand away from VEGFR2. Both VEGF-C and VEGF-D are synthesized as pre-pro-proteins and requires the proteolytic cleavage of both C- and N-terminal regions to produce the fully processed forms which have higher affinity for both receptors - VEGFR-2 and VEGFR-3. VEGFR-1 is may block the mitogenic effects of VEGF. This is further proved by the death of VEGFR-1 null mice at E8.5 which displayed other features such as profound endothelial overgrowth and formation of disorganized vasculature. Subsequently, it was also observed that mice with a homozygous deletion of the intracellular tyrosine kinase domain of VEGFR-1 undergoes normal development, albeit with impaired migration of monocytes. The presence of the extracellular ligand-binding region of VEGFR-1, and its high affinity for VEGF, is thought to be important to promote normal embryonic vasculogenesis and angiogenesis.

Importantly, depending on the type of angiogenesis, VEGFR-1 exerts different roles. VEGFR-1 by antagonizing VEGF, during embryogenesis, regulates negatively physiological angiogenesis and is known to disrupt angiogenesis in some tissues, including the cornea. Nevertheless, in pathological angiogenesis, membrane-bound VEGFR-1 has an altered role, though it performs based on the PLGF co-expression. Regardless of PLGF being inactivated in vivo, mice are proficient of developing normally, nevertheless is characterized by impaired angiogenesis. PLGF signaling via FLT1 is essential in pathological angiogenesis evident by in vivo models. Moreover, via VEGFR-2, PLGF acts in synergy with and augments VEGF mediated angiogenesis. VEGFR-2 regulates proliferation, angiogenesis, haematopoiesis and vascular permeability. At E8.5, Both VEGFR-1 and VEGFR-2 null mice, is embryonic lethal, and is associated with absence of vascular formation mature endothelial cells, and haematopoiesis. Three of the major phosphorylation sites namely Tyr951, Tyr1175 and Tyr1214 are linked with the downstream VEGFR-2 signaling. Mice lacking Tyr1173 are lethal embryonically and shares a similar phenotype to VEGFR-2-/- mice, including decreased hematopoietic progenitors and lack of blood vessels. VEGFR-2 mediated Proliferation can be transduced via activation of extracellular-signal-regulated kinase-1/2 (ERK1/2). In the developing kidney of mice, lack of VEGF bioavailability can produce proteinuria. VEGF acts in a paracrine manner for endothelial cell survival and maintenance of fenestration, but it also confirms the VEGF's autocrine action on epithelial podocytes survival. VEGF via NO and PGI2 production modulates vascular tone and permeability. VEGF, via VEGFR-2, causes eNOS serine 1177 phosphorylation, and NO production. VEGF and PLGF synergistically act in pathological angiogenesis. PLGF is thought to in amplify action of VEGF in endothelial cells. VEGF mechanism in a PKC-dependent manner, via a post-transcriptional induces synthesis of PLGF and amplify its effects on endothelial cells, and acts as a link between PKC inhibition in the angiogenic disorders development and betterment of vascular complications. Consequential to increased soluble VEGFR-1 (sFLT-1) production, decreased activity of VEGF-A results in preeclampsia.(Carmeliet 2000, Maynard, Min et al. 2003, Ahmad and Ahmed 2004). Other than sFLT-1 regulating activity of VEGF (Kendall, Wang et al. 1996), VEGFR-2-mediated proliferation of endothelial cells and tumor growth is regulated negatively by membrane-bound FLT-1 (Ahmed, Dunk et al. 1997, Rahimi, Dayanir et al. 2000, Bussolati, Dunk et al. 2001, Dunk and Ahmed 2001,

Zeng, Zhao et al. 2002). Receptor Heterodimerization usually has been reported in G-protein-coupled receptors and in RTK systems(Prinster, Hague et al. 2005, Barnes 2006).

VEGF-A via NO release regulates vascular tone, which is further dependent on eNOS activation. Also, eNOS phosphorylation and NO release are necessary for VEGF-mediated angiogenesis (Murohara, Asahara et al. 1998, Bussolati, Dunk et al. 2001). VEGF mediated vasorelaxation depends on the VEGFR-1 homodimer and/or the $VEGFR_{1-2}$ heterodimer, and does not need the VEGFR-2 homodimer. Endothelial $VEGFR_{1-2}$ activation also leads to phosphorylation of eNOS and NO release. (Murohara, Horowitz et al. 1998). VEGF-A via negative feedback systems; including release of sVEGFR-1 and NO, regulates its own bioactivity (Ahmed, Dunk et al. 1997, Ahmad, Hewett et al. 2011). Both of these are positively regulated by $VEGFR_{1-2}$ activation. VEGFR-1 negatively regulates endothelial and tumor proliferation mediated by VEGFR-2 (Ahmed, Dunk et al. 1997, Rahimi, Dayanir et al. 2000, Zeng, Zhao et al. 2002). Heterodimerization between subunits of VEGFR-1 and VEGFR-2, negatively regulates cellular response and signaling of VEGFR-2 homodimer and, which may be necessary for endothelial cell angiogenesis and homeostasis. (Cudmore, Hewett et al. 2012).

Vascular endothelial growth factor (VEGF) stimulates angiogenesis in a variety of physiological and pathological processes including corpus luteum formation (2), embryogenesis (1), wound healing (4), tumor growth (3), and compensatory angiogenesis in the heart (5). additionally, *in vivo,* VEGF stimulates vascular permeability (6) and exerts procoagulant activity in endothelial cells and monocytes (8)(Senger, Galli et al. 1983, Clauss, Gerlach et al. 1990, Breier, Albrecht et al. 1992, Brown, Yeo et al. 1992, Plate, Breier et al. 1992, Ravindranath, Little-Ihrig et al. 1992, Barnes 2006). VEGF also attracts peripheral blood monocytes, increases the levels of intracellular Ca (18), and stimulates the production of tissue factor(Clauss, Gerlach et al. 1990) (8). Only one specific binding site has been seen on cells of the macrophage/monocyte lineage, Contrary to the expression of both VEGF receptors in endothelial cells. A high molecular weight band corresponding to a tyrosine-phosphorylated protein was detected Upon VEGF activation(Shen, Clauss et al. 1993) (18).

Based on amino acid and cDNA sequences, Placenta growth factor (PLGF) has been recently described to have a strong homology to VEGF. PLGF is localized in placenta and HUVECs(Maglione, Guerriero et al. 1991) (19, 20). PLGF is a very weak stimulator of endothelial proliferation and chemo- taxis (20), a finding which can be elucidated. By the hypothesis that PLGF exhibits lower affinity to one or both of the two VEGF receptors. Certainly, with extracellular domains from either Flk-1/KDR or FLT-1, when binding competition studies are performed, PLGF appears to bind with FLT-1 but not to KDR/Flk-1(Park, Chen et al. 1994) (Kendall and Thomas 1993)(12, 21).

The close sequence homology between VEGF and PLGF (53% based on amino acid comparison) led to the indication that these polypeptides may mediate biological activities in a similar way. Nevertheless, when VEGF was compared to PLGF in terms of their abilities to induce proliferation of endothelial cells, PLGF was observed to be much less active than VEGF(Maglione, Guerriero et al. 1991) (20, 30). This may be because of the preferential binding of PLGF to FLT-1, in comparison to Flk-1/KDR, as observed in studies using soluble VEGF receptors(Kendall and Thomas 1993, Park, Chen et al. 1994) (12, 21). FLT-1 “knockout” mouse embryos was observed to have defective functionality of vascular tubes, suggesting that FLT-1 plays an key role in endothelium luminal differentiation or endothelium interactions with the extracellular matrix (Fong, Rossant et al. 1995)(33).

4.2.8.2 PLGF

4.2.8.2.1 Cellular activities and molecular mechanisms

Placental growth factor is a member of the vascular endothelial growth factor (VEGF) family, consisting of 4 ligands (VEGF-A, -B, -C and PLGF) and five receptors (VEGFR-1, -2, -3 and NRP1 and NRP2) (Maglione et al. 1991; Hauser and Weich 1993; Cao et al. 1997; Yang et al. 2003). VEGFR-1 and VEGFR-2 are expressed in the cell surface of most blood endothelial cells. In contrast, VEGFR-3 is largely restricted to lymphatic endothelial cells. PLGF is localized in the placental trophoblasts. PLGF is also found in low levels in many other tissues, including the skeletal muscle, heart, thyroid, lung, liver, and bone. The

human PLGF gene is situated on chromosome 14q14 and encodes 4 isoforms. The sequence of PLGF spans an 800-kb long DNA segment comprising seven exons. While 4 isoforms of PLGF has been described in humans, mice only express one isoform equivalent of PLGF-2. Both PLGF-1 and PLGF-3 exclusively bind to VEGFR-1, that is highly expressed in endothelial cells. Both neuropilin-1 (NRP1) and neuropilin-2 (NRP1), are also expressed in endothelial cells, to which PLGF binds. Distinct to VEGF, PLGF binding to VEGFR-1 results in alternative tyrosine residues phosphorylation and gene expression in endothelial cells. PLGF is released as a glycosylated homodimer. While PLGF-2 and PLGF-4 consists of heparin binding domains, PLGF-1 and -3 are diffusible isoforms. A common regulation mechanism appears to be present amongst PLGF1 and 2, since they are secreted in a strongly correlated manner during pregnancy. The presence of a heparin binding domain indicates that PLGF-2 and -4 act in an autocrine manner and remain cell membrane-associated, while the diffusible forms of PLGF affect targets in a paracrine fashion. Yang et al. 2003).

PLGF affects not only endothelial cells but also a whole array of other cell types, and its broad spectrum of pleiotropic activities in various biological processes continues to expand. Unlike VEGF, PLGF redundant for developmental and physiological processes but is more important in diverse conditions such as tissue ischemia, malignancy, inflammation, and multiple other diseases. Precisely because PLGF has a negligible role in health, it has been suggested that PLGF blockade might inhibit these disease processes without affecting normal health. Meanwhile, clinical evaluation of the therapeutic potential of an anti-PLGF monoclonal antibody (mAb) for cancer has commenced. The proangiogenic activity of PLGF relies on direct effects on endothelial and mural cells, as well as on indirect effects on nonvascular cells with proangiogenic activity. PLGF enhances the proliferation, migration, and survival of endothelial cells, although some of these effects remain debated. This cytokine also stimulates proliferation of mesenchymal fibroblasts and regulates the contractile response of mural cells, organized around the endothelium during collateral vessel growth. Moreover, PLGF attracts and activates macrophages, which interferes with dendritic cell differentiation , as well as with antigen recognition, has the capacity to secrete angiogenic and lymphangiogenic factors (Selvaraj et al. 2003), (Lin et al. 2007; Rolny et al. 2011). PLGF induces migration of

keratinocyte in wound healing, increases retinal pigment epithelial cells chemotaxis, recruits mesenchymal progenitors in endochondral ossification. It also promotes axon growth, promotes survival of cortical neurons, and stimulates Schwann cells migration and proliferation. PLGF stimulates hematological and solid tumors growth (Fiedler et al. 2005), (Failla et al. 2000), (Hollborn et al. 2006). (Du et al. 2010), (Cheng et al. 2004), (Chaballe et al. 2011a). (Fischer et al. 2008; Schmidt et al. 2011).

Despite weak tyrosine kinase activity, it was thought initially that membrane-anchored FLT1 functions as a ligand trap, due to its high affinity for VEGF. According to this model, PLGF induce indirectly growth of endothelial cell via VEGF displacement from the FLT1 decoy receptor, thus releasing VEGF for VEGFR-2 activation. Accumulating evidence indicates additional mechanisms. For instance, PLGF increases angiogenic factors expression such as basic fibroblast growth factor (FGF2), VEGF, matrix metalloproteinases (MMPs), platelet derived growth factor b (PDGFB), among other molecules. Moreover, FLT1 activation by PLGF stimulates an intermolecular FLT1: VEGFR-2 cross talk that amplifies VEGF/VEGFR-2 signaling, indicating that endothelial cells have the capacity to enhance their own ability to respond to VEGF by displacing PLGF. PLGF via FLT1 activation, stimulates signaling pathways different from those induced by VEGF. This occurs in macrophages, where FLT1 is expressed exclusively, but also occurs in endothelial cells, which increases FLT1 expression in pathological conditions (Autiero et al. 2003). As demonstrated by mass spectrometry, VEGF and PLGF stimulates phosphorylation of distinct tyrosine residues of FLT1, thus emphasizing that VEGF and PLGF via FLT1 transmit distinct angiogenic signals. Furthermore, it was recently observed that in response to its different ligands, FLT1 transmits distinct intracellular signals, with only VEGF-B but not VEGF or PLGF inducing trans-endothelial lipid uptake. Other studies reported contradictory findings about the PLGF's biological activity, and some of its activity profile remains argued. For example, PLGF has been stated to be ineffective in inducing endothelial cell growth and migration in vitro, a finding recognized to the weak FLT1's tyrosine kinase signal activity. It should be noticed, however, that endothelial cells synthesize abundant PLGF in culture, saturating FLT1 and thereby making it insensitive to exogenous PLGF. Such a hypothesis is supported by findings that PLGF knockout mice's endothelial cells are capable of responding to exogenous PLGF and that loss of PLGF

decreases response to endothelial cells(Carmeliet et al. 2001; Schmidt et al. 2011), (Park et al. 1994). (Roy et al. 2005; Marcellini et al. 2006). (Autiero et al. 2003).

PLGF suppresses tumor angiogenesis when overexpressed to supraphysiological levels in cancer cells that coexpress VEGF. In such scenarios, at the cost of several proangiogenic VEGF: VEGF homodimers, VEGF: PLGF heterodimers are formed, illustrating the reduced tumor angiogenic activity (Eriksson et al. 2002; Xu et al. 2006; Schomber et al. 2007). Likewise, in tumor cells, overexpression of a PLGF variant containing an endoplasmic reticulum retention signal induced VEGF: PLGF heterodimer formation and reduced tumor growth and angiogenesis. Furthermore, a PLGF variant, still capable of heterodimerizing with VEGF, but incapable of binding to FLT1, also decreased VEGF- and PLGF-dependent angiogenesis in tumors (Tarallo et al. 2010). However, the precise role of the VEGF: PLGF heterodimers remains arguable, as proangiogenic but not antiangiogenic effects of these heterodimers have been stated (DiSalvo et al. 1995; Cao et al. 1996a, b; Autiero et al. 2003). Not much is known about the proangiogenic or antiangiogenic activity of VEGF: PLGF heterodimers that are occurring naturally in physiological settings in vivo. It thus remains unclear if the PLGF: VEGF heterodimers' antiangiogenic activity only becomes obvious when PLGF at supraphysiological levels is overexpressed. A proangiogenic effect of PLGF has been observed when it is expressed by stroma or tumor cells endogenously, or when a PLGF transgene is modestly overexpressed by cancer cells (Hiratsuka et al. 2001; Adini et al. 2002; Li et al. 2006; Marcellini et al. 2006; Kerber et al. 2008; Tarallo et al. 2010). Moreover, PLGF's genetic neutralization of PLGF, rather than promoting, inhibits vascular growth in adipose tissue, tumors, and other injured, ischemic, or inflamed tissues.

4.2.8.2.2 PLGF and angiogenesis

PLGF is also not necessary for exercise-induced angiogenesis in the skeletal muscle or heart (Gigante et al. 2004). This redundancy in health is moreover demonstrated by an observation that sFLT-1 trap which neutralizes VEGF, VEGF-B, and PLGF stimulates a similar phenotype as an anti-VEGF antibody in healthy animals (Malik et al. 2006).

Although PLGF is expressed by trophoblast cells and placental villi in pregnancy (Munaut et al. 2008; Depoix et al. 2011), PLGF-deficient mice are fertile (Carmeliet et al. 2001). Nevertheless, increased placental levels of sFLT-1 most likely neutralize local PLGF (Kendall et al. 1996; Cindrova-Davies et al. 2011; Furuya et al. 2011 PLGF also modulates the endometrial uterine natural killer cells maturation required for trophoblast invasion (Tayade et al. 2007). Thus, though during development, PLGF is expressed, its role is largely redundant. Placental growth factor is pro-angiogenic as it stimulates VEGF's activity by binding competitively to the VEGFR-1 receptor, thus causing VEGF to bind to VEGFR-2 which has potent tyrosine kinase activity. Nevertheless, PLGF also exerts its effect via other mechanisms such as VEGFR-2's intermolecular transphosphorylation following VEGFR-1 activation, which amplifies response of VEGFR-2 to binding of VEGF. In addition, PLGF forms a heterodimer with VEGF, which may either have pro- or anti-angiogenic effects (Autiero et al., 2003). PLGF-deficient mice exhibit normal health, development, viability, indicating that endogenous PLGF may not be necessary for vascular development (Carmeliet et al., 2001).

In normal endothelial cells, rather than vasculogenesis, PLGF most likely may influence angiogenesis by amplifying VEGF's effects. Nevertheless, PLGF and FLT1 play a role in the mesenchymal endothelial precursor cells' mobilization that contribute to vasculogenesis (Li *et al*. 2006). PLGF also induces release of NO (Bussolati et al., 2001) and prolongs capillary networks' survival and stability (Cai et al., 2003). Studies in vivo have observed that during new vasculature formation in the adult, endothelial cells become more responsive to VEGF via PLGF and VEGFR-1 upregulation. PLGF binds to FLT1 and directly affects endothelial cells, thereby stimulating its own signaling as well as amplifying VEGF-mediated angiogenesis (Carmeliet et al., 2001). PIGF also appears to be involved in local

tissue specific homeostasis of endothelial cells, where VEGFR-1 activation appears protective against liver toxicity via inducing local tissue specific growth factors release (LeCouter et al. 2003).

The explanation possibly for the significant role of PLGF in tissues other than the placenta is angiogenesis in response to pathological injury or ischemia is that its levels or expression is undetectable or low in most of the healthy tissues, but significantly up-regulated in pathologies (Marrony et al. 2003; Fischer et al. 2008). In line herewith, the expression of FLT-1 is also up-regulated in disease (Fischer et al. 2008). Knockout mice (PLGF−/−) have defective arteriogenesis and angiogenesis during pathological conditions such as limb, heart and ocular ischemia. Physiological exercise induced ischemia in cardiac and skeletal muscle does not induce PLGF synthesis but upregulation of PLGF is seen in pathological conditions characterized by aberrant angiogenesis such as cancer , coronary artery disease, and diabetes (Carmeliet et al., 2001). Expression of PLGF is part of the angiogenic switch that supports vascularization of tumor. Angiogenesis and growth in tumor is inhibited in transgenic mice bearing truncated/signaling-inactive VEGFR-1 or PLGF null mice.

PLGF deficient mice demonstrated significantly decreased neovascularization and growth (Carmeliet et al., 2001). There appears to be a positive correlation between PLGF expression and protein and cancer severity, with an inverse relationship between PLGF and survival. There appears to be a positive correlation between PLGF expression and protein and cancer severity, with an inverse relationship between PLGF and survival. (Cai et al., 2003). nevertheless, unlike overexpressed VEGF in most tumors, increased expression of PLGF occurs in only a few cancers such as a subset of meningiomas, melanomas, hypervascular renal cell carcinomas (Lacal et al., 2000), (Donnini et al., 1999).

PLGF expression in heart induces wound healing (Iwama et al., 2006) and after acute myocardial infarction, improves performance of heart (Roncal et al., 2010) PLGF appears to induce collateral growth in the ischemic heart and limb (Pipp et al., 2003, Luttun et al., 2002, Kolakowski et al., 2006). On the other side, PLGF is involved in inflammatory angiogenic disorders such as arthritis or atherosclerosis (Pilarczyk et al., 2008) (Yoo et al.,

2009). Since macrophages and monocytes express VEGFR-1 receptors, PLGF promotes inflammation and acts as a chemoattractant by increasing expression of tissue factor production of cytokines and migration of monocytes (Clauss et al., 1996), (Fu et al., 2009) (Carmeliet et al., 2001). In pathological conditions, the specific PLGF expression presents itself as a good opportunity for targeted therapy. In patients with recurrent glioblastoma multiforme, in combination with anti-VEGF antibody bevacizumab , A phase 1 study of an anti-PLGF monoclonal antibody did not demonstrate improved survival.6 studies have further been conducted in patients with metastatic colorectal or ovarian cancer and hepatocellular carcinoma but results have not been published.

4.2.8.2.3 PLGF, redundant in development and health

Given its pleiotropic activities, it is not surprising that PLGF stimulates different biological effects in vivo. PLGF-deficient mice display normal health, development, viability, suggesting that endogenous PLGF is unessential for homeostasis and vascular development in the adult (Carmeliet et al. 2001). PLGF is also not necessary for exercise-induced angiogenesis in the skeletal muscle or heart (Gigante et al. 2004). This redundancy in health is further demonstrated by the fact that like an anti-VEGF antibody in healthy animals, a similar phenotype is induced by a sFLT-1 trap that neutralizes VEGF, VEGF-B, and PLGF (Malik et al. 2006). A possible explanation for the more important pathological role of PLGF is that its low expression in healthiest tissues, but up-regulated significantly in pathologies (Marrony et al. 2003; Fischer et al. 2008). In line herewith, expression of FLT1 is also increased in pathologies (Fischer et al. 2008). During pregnancy, although expression of PLGF is seen by trophoblast cells (Munaut et al. 2008; Depoix et al. 2011), PLGF-deficient mice are fertile (Carmeliet et al. 2001). Nevertheless, increased levels of sFLT-1 in the placenta most likely neutralize local PLGF (Kendall et al. 1996; Cindrova-Davies et al. 2011; Furuya et al. 2011). PLGF also modulates endometrium's uterine natural killer cells maturation required for trophoblast invasion (Tayade et al. 2007). Overall, during development, although PLGF is expressed, its role is largely redundant.

4.2.8.2.4 PLGF in reproduction

In reproduction, the role of PLGF is still remains to be understood. Since PLGF knockout mice are fertile and pups have similar growth potential compared to wild type mice, PLGF is thought to be redundant in reproduction. (Binder, Evans et al. 2016). Nevertheless, during the secretory phase of the human menstrual cycle, endometrial tissue has been reported to release PLGF. The presence of PLGF during this window supports a role of PLGF in influencing implantation of embryo, but this has yet to be further illustrated. differences in the development of adult and fetal brain have recently been reported, despite PLGF knockout mice appear to be normal. (Luna, Kay et al. 2016). The preliminary data in the children of women with preeclampsia indicates mild differences in vascular development of brain, which appears to be related to intrauterine events. Therefore, despite PLGF appear not to be required to reproduction, it is still likely to be an important influence on vascular development in pregnancy(Dang, Croy et al. 2016).

4.2.8.2.5 The role of PLGF in placental development

Maternal PLGF concentrations is prominently increased during gestation. PLGF promotes the maturation and development of the placental vascular system. Implantation sites of PLGF knockout mice show abnormal placental vasculature(Ratsep, Carmeliet et al. 2014).https://www.nature.com/articles/jhh201761 - ref10 There is increased lacunarity and reduced branching in the anti-mesometrial (feto-placental) vessels, suggesting a lack of uniformity in the formation of vasculature. Utero-placental vasculature shows reduced branching, but decidual invasion remains unaffected. Lymphatic vessels of uterus of mouse are also abnormally developed. In human placenta, PLGF expression corresponds with different stages in placental development with maturation of the utero-placental circulation and non-branching angiogenesis of the feto-placental circulation coinciding with increased PLGF expression during later stages of pregnancy. Distinctly, placental circulation development is influenced by PLGF even though the lack of PLGF does not lead to the death in offspring of knockout mice. (Kang, Park et al. 2014). In contrast, a mouse knock-in with PLGF expressed in T cells showed reduced angiogenesis in offspring,

suggesting that, the effect of PLGF in pregnancy is specific to situation corresponding to the alterable role of PLGF in a life cycle of tumor. When hypothesizing possible effects in human pregnancy from the findings in rodent studies, differences in physiology of placenta between species must be illustrated. The effects of aberrant placental development may not be readily evident in mice, despite abnormal implantation apparent in PLGF knockout mice may not lead to embryonic or fetal death. Contrast to human pregnancy, placenta in mouse does not invade into the myometrium and limited endovascular invasion is seen. As a consequence, abnormal remodeling of mice's spiral arteries does not lead to insufficiency of placenta or abnormal regulation of blood pressure. (Burke, Barrette et al. 2010). Processes in the development of human placenta may be more extensively facilitated by PLGF. PLGF is expressed in placenta profoundly from the second trimester when the utero-placental circulation is advancing, beginning at 16–18 weeks' gestation, with remodeling of myometrial spiral arteries in a 'second wave' of invasion. However, there have been contradictory reports as to whether PLGF causes invasion of trophoblasts (Athanassiades and Lala 1998, Knuth, Liu et al. 2015). Trophoblast acquire invasive characteristics in response to high oxygen tension and expression of PLGF also increases with improved oxygenation of placenta. It is however uncertain whether these two events have a directly linked regulatory mechanism. In non-trophoblast cells, hypoxia upregulates PLGF expression. In contrast, normoxia increases and hypoxia suppresses transcriptional activity of PLGF in trophoblast thus indicating a specific regulatory function and mechanism in these cells. PLGF influences uterine natural killer cells Differentiation and these cells may in turn facilitate trophoblast invasion into the decidua. (Tayade, Hilchie et al. 2007). PLGF increases trophoblast's proliferation. PLGF decreases trophoblast apoptosis when they are starved, but not when subjected to inflammatory cytokines(Desai, Holt-Shore et al. 1999, Arroyo, Price et al. 2014). This may manifest as enhanced circulating trophoblast debris found in cases of pre-eclampsia (which is associated PLGF deficiency), but the exact role of PLGF mediated reduction of apoptosis in the development of placenta is not clear.

4.2.8.2.6 PLGF levels in normal pregnancy

While generally, maternal total VEGFA levels gradually increase throughout pregnancy, low Concentrations of PLGF are found in the first trimester of an uncomplicated pregnancy and rises from week 11 to 12 onwards to a peak at week 30, after which it reduces. This is contrary to sFLT-1, which elevates towards the end of gestation. This normal discrepancy of levels of angiogenic factors occurs as the bioavailability of PLGF is decreased by binding to sFLT-1. Normal concentrations of PLGF are dependent on gestational age, with the lower limit of normal (defined as the 5th centile) ranging from a peak of approximately 141 pg ml−1 at around 30 weeks' pregnancy to 23 pg ml−1 at term. Maternal plasma levels of PLGF gradually rise during gestation to reach a peak at ~30 weeks of pregnancy. These profiles are altered in pre-eclampsia, where concentrations of PLGF are comparatively reduced throughout gestation. The expression of PLGF in placenta exponentially increases from the mid pregnancy, corresponding with non-branching angiogenesis of feto-placental vessels and maturation of the utero-placental blood flow. Placental growth factor may contribute to increased proliferation, invasion and reduced apoptosis of trophoblasts(Saffer, Olson et al. 2013).

4.2.8.2.7 PLGF in pre-eclampsia

Urinary and Serum PLGF is observed to be decreased in women both during the time of diagnosis with pre-eclampsia and well in advance of onset of syndrome. The PLGF deficiency is possibly due to a combination of reduced PLGF expression and free PLGF due to sFLT-1 binding, which is increased in affected women. (Levine, Maynard et al. 2004). In early pregnancy, lower concentrations of PLGF are seen in women who subsequently develop preeclampsia than those with normal pregnancy, but levels of sFLT-1 are no different, indicating that expression of PLGF in the placenta is reduced. However, towards end of pregnancy, a reciprocal relationship between sFLT-1 and PLGF is observed with increased total (free and bound to VEGF or PLGF) sFLT-1 and decreased free PLGF levels(Maynard, Min et al. 2003). This indicates that low PLGF levels occur in the latter half of pregnancy, predominantly due to PLGF sequestration by sFLT-1.

Reduced PLGF concentrations is possibly both a contributing factor to continued abnormal growth during the latter half of pregnancy and a consequence of abnormal early events in placentation. The hypothesis that PLGF is an indicator of abnormal placentation is supported by the observation that women without pre-eclampsia who give birth to small for gestational age babies also have reduced PLGF concentrations early in pregnancy(Poon, Zaragoza et al. 2008). The data regarding the PLGF expression in placenta is contradictory. PLGF expression is hypothesized to be decreased due to suppression by consistent placental hypoxia occurring due to an immature utero-placental blood flow. However, studies have also observed in pre-eclamptic placental tissue, no change or increase in expression of PLGF(Khaliq, Dunk et al. 1999, Hoeller, Ehrlich et al. 2017). Although regulation of PLGF expression is ambiguous, several mechanisms have been explored such as epigenetic changes altering the effect of the hypoxia-inducible factor-1α (HIF1-α) and endoplasmic reticulum stress. The role of HIF1-α in growth of trophoblasts is debated. Inflammation may also influence expression of PLGF as concentrations of PLGF are increased in sepsis(Gobble, Groesch et al. 2009).

4.2.8.2.8 PLGF for the prediction and diagnosis of pre-eclampsia

The differences in the levels of circulating angiogenic factors between normal and pre-eclamptic pregnancies has led to the investigation into whether these factors can recognize women who require close monitoring. The only therapy for preeclampsia is the delivery of the placenta. Several studies of PLGF testing have focused on either confirmation of the diagnosis or prediction of preeclampsia, once preeclampsia is suspected, included in the review by Kleinrouweler et al (Kleinrouweler, Wiegerinck et al. 2012). PLGF has been demonstrated to be an influential component of prediction of preeclampsia at 11 to 13 weeks. (Akolekar, Syngelaki et al. 2011). . A systematic review indicated that prediction of preeclampsia may improve by inclusion of this biomarker into a clinical multivariable model and another reported that PLGF is cost-saving if utilized before 35 weeks of pregnancy for predicting preeclampsia requiring delivery within a specified time (Frampton, Jones et al. 2016). In women with suspected pre-eclampsia, but not yet satisfying diagnostic criteria, the plasma PLGF alone or sFLT-1: PLGF ratio is

beneficial as a ‘rule out’ test with a high negative predictive value. Maternal serum PLGF levels less than the 5th centile for pregnancy during the presentation time did better than combination of 5 factors that are commonly used clinical parameters (systolic and diastolic blood pressure, uric acid, alanine transferase, and dipstick proteinuria) (ROC area 0.87 vs 0.70 *P*<0.001) in identifying women with pre-eclampsia necessitating delivery within 2 weeks. (Chappell, Duckworth et al. 2013, Chau, Hennessy et al. 2017). low PLGF reflects placental disease which is further supported by the fact that low PLGF’s sensitivity was highest for the small for gestational age delivery. from studies reporting the PLGF usage as a prognostic test for women with suspected or confirmed preeclampsia, a systematic review of the findings found that , in women with hypertensive disorders of pregnancy, PLGF could be a marker for the preterm delivery’s prediction, due to maternal and fetal indications(Ukah, Hutcheon et al. 2017).

In prediction of pre-eclampsia, the angiogenic factors’ utility such as PLGF, VEGF, and sFLT-1 is limited likely by the heterogeneity of pathology that triggers the spectrum of clinical presentation of pre-eclampsia. Affected women range from those with mild symptoms presenting at term to others with early-onset disease and severe intrauterine growth restriction. Severe, early disease is in strong association with abnormal placentation and abnormal production of angiogenic factors are more pronounced in these patients. Abnormal sFLT-1: PLGF ratio and consistent low PLGF levels throughout pregnancy recognizes a subset of women with an early and more severe presentation of the disease. The angiogenic factors may be utilized in classifying patients with preeclampsia to promote more directed research specific to pre-eclampsia subtypes Chau, 2017 #583}.

4.2.8.3 sFLT-1

The human placenta is a vascular organ and its function is to develop a large vascular network (thus, being a site of active angiogenesis and vasculogenesis) allowing for nutrient, waste, and gas exchange between the fetal and maternal circulations. Indeed, the placenta produces a variety of angiogenic factors such as vascular endothelial growth

factor (VEGF), placental growth factor.(Ahmed, Dunk et al. 2000), alongside with their receptors FLT-1 and KDR (Clark, Smith et al. 1998). Pseudovasculogenesis is the process where Cytotrophoblasts invade the maternal spiral arterioles and acquire properties of endothelial cells (Zhou, Ahmad et al. 2007). Vascular remodeling depends on a fine balance between antiangiogenic and proangiogenic factors modulate. VEGF stimulates angiogenesis interacting with 2 high-affinity receptors, VEGF receptor-1 (VEGFR-1, also known as FLT-1) and VEGF receptor-2 (VEGFR-2, also known as KDR/Flk-1). In the final stage of the gestation, when continued placental growth is no longer required, the production of different antiangiogenic factors is promoted such as a soluble form of VEGFR-1 (sFLT-1). sFLT-1 sequesters extracellular VEGF and placental growth factor, inhibiting their angiogenic functions. VEGF with its receptors is fundamental for the physiological development and function of the placenta as well as for promotion and maintenance of the established maternal vasculature. VEGF is not uniquely expressed by placenta and endothelium. It also expressed by different cells, e.g., smooth muscle, monocytes/macrophages, and poly- morphonuclear neutrophils (PMNs). VEGF acts through Fms-like tyrosine kinase 1 (FLT-1)/ VEGF receptor-1 and VEGF receptor-2 (FLK-1/KDR(Luttun, Autiero et al. 2004, Krysiak, Bretschneider et al. 2005).

The full-length RNA coding for FLT1 contains 30 spliced exons and is translated into a ∼200 kDa transmembrane protein with an extracellular N-terminal ligand-binding domain, a single membrane-spanning segment, and a C-terminal intracellular segment presenting two tyrosine kinase domains. Soluble VEGFR-1/sFLT-1 presents the ligand-binding domain (the first 13 exons, 687aa) as well as the full-length VEGFR-1/FLT1, while, in lieu of the membrane-spanning and C-terminal domains, (Kendall and Thomas 1993). sFLT-1 contains a 31 amino acid C-terminal sequence (Maynard, Venkatesha et al. 2005).

sFLT-1 consists of 6 immunoglobulin-like domains, with a binding site for VEGF and PIGF within the second domain from the N-terminus.[(Kendall and Thomas 1993 Besides sFLT-1, other soluble forms have also been reported {Sela, 2008 #540, Thomas, Andrews et al. 2009) . sFLT-1 can effectively dimerize, but the absence of a kinase domain makes tyrosine phosphorylation impossible upon ligand binding(Thadhani, Kisner et al. 2011). As a result, sFIt-1 effectively sequesters agonists of FIt-1, and is involved in the regulation

of this receptor in tissues such as kidney, liver, and brain(Maynard, Epstein et al. 2008).The theoretical molecular weight of sFLT-1 is around 78 kDa, but the experimental size range is 85–120 kDa. Indeed, sFLT-1 is secreted as a ∼100 kDa protein, that by binding circulating VEGF and PLGF antagonizes their functions.

Initially it was proposed that low levels of VEGF in maternal decidua may contribute to preeclampsia condition because of aroused levels of endogenous sFLT-1, which may antagonize the beneficial effects of VEGF in this disorder(Luft 2014).The main source of sFLT-1 during pregnancy is thought to be the placenta even though also endothelial cells and monocytes contribute to its production(Kendall and Thomas 1993, Maynard, Min et al. 2003) (Maynard, Venkatesha et al. 2005) sFLT-1 mRNA is highly expressed in the placenta, and a drastic decrease of serum concentration of sFLT-1 follows delivery of the placenta (Hornig, Barleon et al. 2000, Koga, Osuga et al. 2003). There exist different isoforms of sFLT-1 and sFLT-1 e15a is the one mainly transcribed from FLT-1 within the placenta (Jebbink et al. 2011). While sFLT-1- e15a is placental specific, it also appears specific to humans and higher order primates(Sela, Itin et al. 2008, Thomas, Andrews et al. 2009).). sFLT-1 i13 is secreted by different types of cells, in particular by the endothelial ones. Hence, its serum levels of sFLT-1 i13 could increase in other maternal inflammatory conditions in the absence of preeclampsia, such as obesity and gestational diabetes mellitus

FLT-1 gene splicing and therefore its expression is influenced by several factors such as short-term regulation by oxygen, nitric oxide levels, and genetics. Notably, hypoxic conditions induces sFLT-1 expression. In physiological pregnancy conditions, the placenta development occurs in a hypoxic environment, triggering a significant increase in sFLT-1 expression(Shibata, Rajakumar et al. 2005). In early-onset preeclamptic patients, this increase is predicted to be up to 43 times more pronounced. In a rat model of preeclampsia impairment of nitric oxide signaling has been associated with serum sFLT-1 increase and possibly constitutes a secondary factor contributing to sFLT-1 trend of expression in human preeclampsia.[(Sela, Itin et al. 2008, Thomas, Andrews et al. 2009).

The VEGFR-1 (FLT-1) gene has 2 mRNAs: one is a long form of approximately 8 kb, and the other is a short form of 2.5 to 3.0 kb. (Shibuya, Yamaguchi et al. 1990). in normal

placenta, the short mRNA is expressed highly, encoding a soluble form of FLT-1 known as sFLT-1. The sFLT-1 consists of 6 Ig-like domains with a short, 31 amino acid–long tail derived from the 5′ region of intron 13 and demonstrates a strong binding ability to VEGF-A, PLGF, and VEGF-B(Kendall and Thomas 1993, Kondo, Hiratsuka et al. 1998)

4.2.8.3.1 The many variants of sFLT-1

sFLT-1 occurs due to alternative splicing of the FLT-1 pre-mRNA and in recent years multiple sFLT-1 splice variants have been recognized. The importance of these different splice variants remains unknown. Alternative splicing is comparatively new concept, but has been shown to occur in more than 80% of genes in the human genome. The majority of splicing events that are affecting the coding sequence, are regulated by cis and trans-elements. The former are sequences within the pre-mRNA that assist to direct splicing. These include intronic counterparts (ISE's and ISS's), exon splicing enhancers or silencers (ESE's or ESS's respectively), as well as polyadenylation signals within the sequence. Trans-elements are cellular factors, that can be either RNA or protein that controls splicing. A family of proteins named for the long repeats of serine (S) and arginine (R) residues present. They act in both the regulation and performance of splicing.
(He, Smith et al. 1999, Matlin, Clark et al. 2005, Chasin 2007, Palmer, Tong et al. 2017).

The FLT-1 gene encodes four soluble alternative sFLT-1 splice variants and the full-length membrane bound FLT-1 receptor (VEGFR-1). As can be demonstrated all transcripts are similar to exon 13, with the soluble transcripts each recognized by a unique C-terminal region. Of these, sFLT-1 i13, sFLT-1 v4 and sFLT-1 e15a are translated into protein with only the former two being increased in pre-eclampsia(Thomas, Andrews et al. 2009). The FLT-1 gene encodes an mRNA transcript consisting of 30 exons. The FLT-1 pre-mRNA has been reported to produce four truncated soluble splice variants and full-length membrane bound FLT-1. Of these, three sFLT-1 variants were thought to be translated into protein, two of which are highly expressed in pre-eclamptic placenta. (Heydarian, McCaffrey et al. 2009).

These two variants will be referred to as sFLT-1 i13 (also known as sFLT-1; sFLT-1_v1) and sFLT-1 e15a (also known as sFLT-1_v2; sFLT-1 14). While differing only at their C-terminus,

these splice variants share sequence homology significantly with the full-length FLT-1 receptor. sFLT-1 i13 was the first sFLT-1 recognized and consists of the first 13 exons of FLT-1 with an exon 13 extension into the intronic sequence because of a read-through of the exon 13-14 splice site. Intron 13 consists of two widely separated polyadenylation signal sequences that are cis-elements controlling the alternative splicing of the i13 variant. This synthesizes either a short or long 3'-untranslated region (UTR) depending on which polyadenylation sequence is utilized. The consequence of alterations in the 3'-UTR are unknown as of now, but have been indicated to possibly modulate expression of mRNA in a temporal or tissue-specific fashion, or regulate mRNA stability or its translational efficiency(Thomas, Andrews et al. 2007) . Consequently, after 657 amino acids, the i13 protein deviates from full length FLT-1. i13 producing an 85-95 kDa protein, encodes a distinct 31 amino acid C-terminal tail (not present on FLT-1). Comparatively, sFLT-1 e15a was recognized more recently. This variant is more specific to higher order primates and humans, and is a consequence of insertion of an AluSq sequence into the primate genome following a retro transposition event that occurred around 40 million years ago. sFLT-1 e15a ends within an alternatively spliced exon 15a. This encodes a unique polyserine tail and is followed proximally by an AluSq sequence. This is a 3'-poly(A) tail which is analogous to the majority of Alu sequences. (Thomas, Andrews et al. 2007) observed that it has two polyadenylation signal sequences which causes cleavage of mRNA. (Sela, Itin et al. 2008) also recognized the presence of this novel sFLT-1e15a variant (which they named sFLT-1 14). They were able to show that it begins from using a previously unknown splice acceptor site within intron 14 synthesizing a protein made up of the first 14 exons of FLT-1 followed by a 480 nucleotide stretch of intronic sequence. This encodes the unique exon 15a followed by a 3'-UTR containing the complete AluSq sequence. Thus, this splice variant encodes a protein that shares 706 amino acids with FLT-1 and has a unique 28 amino acid tail, synthesizing a protein that is heavily glycosylated and 95-135 kDa in size. Notably, except humans and higher order primates, sFLT-1 e15a is not present from all mammals, emphasizing it as possibly the variant underlying pre 22 Alu sequences were inserted into the primate genome by retrotransposons. It now denotes between 6-13% of human genomic DNA. AluSq is one amongst 12 identified sub-families, and has been inserted approximately 44 million years ago. (Thomas, Andrews et al. 2009).They are seen commonly within intronic and non-coding DNA, however, are capable of affecting transcription of genes. They consist of

many stop codons and can also affect splicing to synthesize truncated proteins. Through their impact on splicing they have been linked to a number of human diseases, such as Alport syndrome ,neurofibromatosis, and familial hypercholesterolemia (Mighell, Markham et al. 1997) .

Within the placenta, trophoblasts situated between the maternal and fetal compartment express sFLT-1 preferentially with the possibility that sFLT-1 acts as a biochemical barrier between maternal and fetal compartment in the placenta by inhibiting excess angiogenesis and abnormal vascular permeability. VEGF's overtrapping may lead to severe problems in the placental circulation, thus the sFLT-1 level at physiological range would be suitable. Interestingly enough, abnormal placental SFLT-1 overexpression was seen in diseases such as preeclampsia.

Although it was earlier thought that sFLT-1 was restricted to the maternal circulation, recent studies have demonstrated non pregnant women has the detectable concentrations of sFLT-1, of monocytes and endothelial origin. (Major, Cambell et al. 2014). This suggested that sFLT-1 might be a regulator of VEGF bioavailability, which is of importance as continuous low VEGF concentrations are required for proliferation and survival of endothelial cells. It has been suggested that sFLT-1 via its heterodimerisation with VEGF receptors may regulate the VEGF bioavailability, hence eliminating VEGF-mediated signal transduction.

In the placenta and serum of pregnant women with preeclampsia, the existence of sFLT-1 in vivo was confirmed subsequently. (Clark, Smith et al. 1998) showed that the sFLT-1 production antagonized VEGF's actions and high concentrations of VEGF was seen in these women.

Furthermore, Levine *et al*. observed an intimate relationship between the degree of preeclampsia and plasma concentrations of sFLT-1. (Levine, Maynard et al. 2004). This strongly indicates that abnormal VEGF-A suppression by sFLT-1 results in hypertension and proteinuria. Maynard *et al*. further reported that in pregnant rats , an artificial sFLT-1 expression with a vector system causes symptoms such as hypertension and proteinuria, suggesting that sFLT-1, partially, causes preeclamptic syndromes(Koga,

Osuga et al. 2003, Maynard, Min et al. 2003). . in mice, a podocyte-specific knockout of the *VEGF-A* gene showed loss of glomerular microvasculature and proteinuria. Thus, in preeclampsia, a severe decrease and block in the VEGF-A concentrations in the kidney due to overexpressed sFLT-1 may lead to proteinuria and glomerular dysfunction. Interestingly, cancer patients under anti–VEGF-VEGFR therapy demonstrated similar symptoms (hypertension and proteinuria).

Expression of sFLT-1 appears to be mainly regulated by hypoxia, such as in the case of preeclampsia. Nevertheless, its expression is also influenced in a tissue-specific fashion. sFLT-1 is expressed in corneal epithelial cells, indicating that sFLT-1 maintains the corneal avascularity and transparency of the eye and inhibits angiogenesis near the lens. (Ambati, Nozaki et al. 2006). sFLT-1 also aids in impermeability of blood brain barrier. Moreover, expression of sFLT-1 is also developmentally regulated, where it plays a key role information of early placenta, with persevering low-grade expression throughout normal pregnancy. So, while sFLT-1 in a spatial and tissue-dependent fashion is expressed physiologically, the mechanisms that control its expression remain unknown. While many studies have recognized hypoxia as the key factor in pathophysiology of pre-eclampsia, that contributes to the increased expression of sFLT-1, the cellular mechanism through which hypoxia produces this effect is yet to be established. HO-1 has been recognized as a possible candidate. In response to hypoxia, the expression of HO-1 is downregulated, where its reduction is associated with rise in sFLT-1. Moreover, enhanced production of anti-angiogenic protein appears to occur due to decreased activation of AKT potentially secondary to decreased HO-1 expression in pre-eclamptic placenta. This pathway nevertheless fails to explain the differential expression patterns of splice variant observed within the pre-eclamptic placenta(George, Colson et al. 2012).

The oxygen sensing jumonji domain containing protein 6 (Jmjd6) was observed to regulate FLT-1's splicing pattern in endothelial cells. Under normoxia, Jmjd6 was able to apply its normal enzymatic functions and hydroxylate a component of the splicing machinery, U2 small nuclear ribonucleoprotein auxillary factor 65-kilodalton subunit (U2AF65). This leads to the splicing machinery generating the full-length membrane-bound FLT-1 transcript. Boeckel et al (2011) showed that under the influence of hypoxia, Jmjd6 was rendered inactive and thus unable to hydroxylate U2AF65. Consequently, the splicing machinery synthesizes a shorter alternatively spliced sFLT-1 transcript

instead(Boeckel, Guarani et al. 2011, George, Colson et al. 2012). Both sFLT-1 variants of appear bioactive since they are capable of antagonizing its actions by binding to VEGF. Given that pre-eclampsia appears to be exclusive to humans, compared to i13, the e15a variant is the predominant isoform in human placenta. It is the main variant in rhesus monkey placenta. Moreover, sFLT-1 e15a appears to be expressed by the human placenta almost exclusively where its expression is ≥600-fold higher than in the other organ systems. sFLT-1 e15a is the predominant isoform of FLT-1 mRNA in placenta, comprising >80% of transcripts compared to sFLT-1 i1(Palmer, Tong et al. 2017).

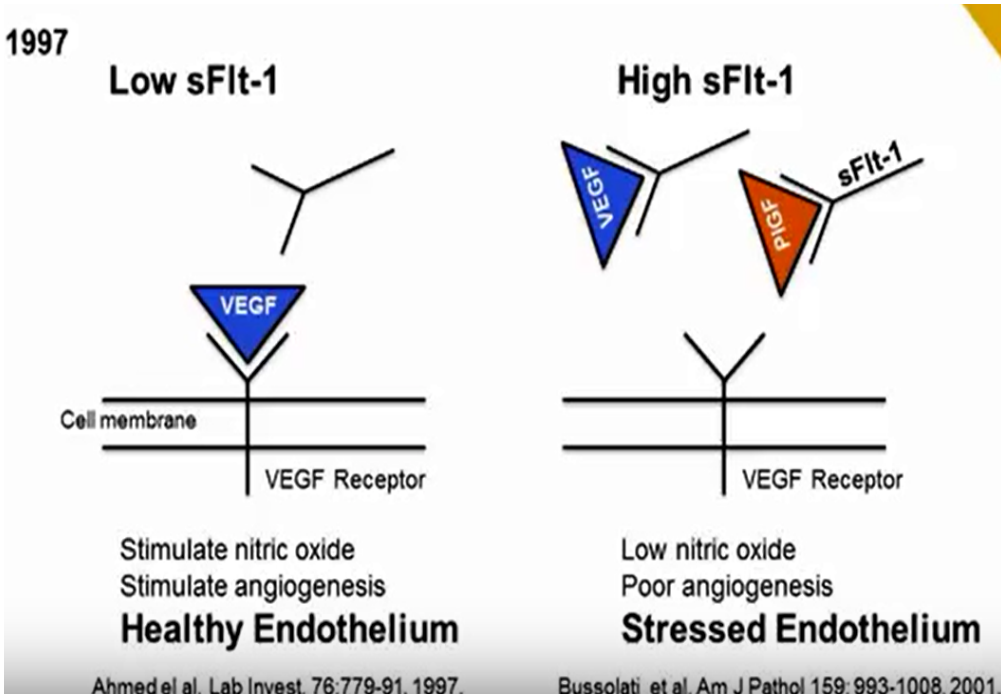


Figure 2

sFLT-1 is an inhibitor of VEGF and PLGF activity

4.2.9 **Signaling regulation of placental angiogenesis**

4.2.9.1 The MAPK pathways

The mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved signal transduction cascades involved in the regulation of different cellular responses such as proliferation, differentiation and cell death. In vertebrates, different isoforms of MAPK have been identified and classified into three subfamilies, i.e., the extracellular signal-regulated kinases (ERKs), p38MAPK, and the Jun N-terminal kinases (JNKs) or stress-activated protein kinases. The MAPK signaling transmits extracellular signals including growth factors, hormones, and chemokines, etc., into the intracellular targets for the vast majority of the fundamental cellular processes. The p38MAPK subfamily includes p38α/MAPK14, p38β/MAPK11, p38γ/MAPK12, and p38δ/MAPK13 (Cargnello and Roux 2011). Targeted disruption of the p38α gene leads to in embryonic mice death at mid-gestation due to severe placental defects (Adams, Porras et al. 2000, Mudgett, Ding et al. 2000). p38α-knockout mice, show placental defects around E10.5, which is evidenced by sparse vascularization and sustained cell apoptosis in the labyrinth layer (100)(Mudgett, Ding et al. 2000). An essential role of P38α in mouse placental development and angiogenesis has been confirmed by specific placental expression of p38α using lentiviral gene delivery technology. Introduction of p38α into p38α-knockout mouse placenta, rescues the embryo of the mutant mice which presents a normal placental vascularization (Okada, Ueshin et al. 2007)(Yamamoto, Flannery et al. 1998, Hatano, Mori et al. 2003) .

4.2.9.2 The PI3K/AKT pathways

The V-AKT murine thymoma viral oncogene homolog 1 (AKT1) family of kinases has three isoforms (e.g., AKT1, 2, and 3), that are encoded by distinct genes In mammals, following induction with growth factors, hormones, and cytokines, etc., activation of phosphotidylinositol-3-kinase (PI3K) phosphorylates phosphatidylinositol 4,5-bisphosphate [Ptdlns(4,5)P2] at the D-3 position of the inositol ring to produce PtdIns(3,4,5)P3, which is then converted to PtdIns(3,4)P by 5'-phosphatase (Vanhaesebroeck, Leevers et al. 1997). The activation process of AKT by phosphorylation is initiated by low micromolar concentrations of Ptdlns(3,4,5)P3 or Ptdlns(3,4)P2 interaction (Alessi, James et al. 1997). Activated AKT can phosphorylates the B-cell CLL/lymphoma (Bcl-2)-associated death promoter (BAD) which interacts with the Bcl family member BclxL, thus blocking cellular apoptosis, phosphorylate glycogen synthase kinase-3 and 6-phosphofructo 2-kinase that are essential for insulin signaling and protein synthase. (Cross, Alessi et al. 1995, Zha, Harada et al. 1996, Deprez, Vertommen et al. 1997). AKT1 disruption leads to significant growth retardation in mice AKT1-null mouse placentas exhibits significant hypotrophy, with decidual basalis reduction. Moreover, the placentas also displays reduced vascularization, leading to placental insufficiency, reduced fetal growth and neonatal mortality (Chen, Xu et al. 2001, Cho, Thorvaldsen et al. 2001, Yang, Tschopp et al. 2003). eNOS-NO and AKT1 but not AKT2 and AKT3 are essential for placental angiogenesis. This is further supported by the fact that placentas of the AKT1-null pregnant mice are associated with significantly decreased AKT1 and eNOS phosphorylation. AKT2-null mice display a type-II diabetes-like syndrome and mild growth-retardation and age-dependent loss of adipose tissue (113). AKT3 appears to be important in postnatal brain development (Yang, Tschopp et al. 2003),(Woulfe, Jiang et al. 2004, Easton, Cho et al. 2005).

Several studies reported that activation of the MAPK (ERK1/2, JNK1/2, and p38MAPK), PI3K/AKT1, and eNOS/NO pathways is very important/necessaire for VEGF- and FGF2-induced angiogenesis in diverse endothelial cells. It has been demonstrated that in placental endothelial cells, activation of the MAPK pathways play an important for the differential regulation of placental endothelial cell proliferation, migration and tube formation (i.e., in vitro angiogenesis) in upon VEGF and FGF2 stimulation in vitro (Cho, Thorvaldsen et al. 2001, Mata-Greenwood, Liao et al. 2008, Feng, Liao et al. 2012, Feng, Zhang et al. 2012).). Inhibition of the ERK1/2 pathway moderately reduces the FGF2-stimulated cell proliferation, while it completely inhibits VEGF-stimulated cell proliferate and VEGF- and FGF2-stimulated cell migration (Zeng, Zhao et al. 2002, Liao, Feng et al. 2009, Feng, Liao et al. 2012, Feng, Zhang et al. 2012). Cell migration stimulated by VEGF also involves stress fiber formation and focal adhesion via the tyrosine kinase Src-mediated phosphorylation of the small actin binding protein cofilin-1 and FAK kinase (Liao, Feng et al. 2009). Inhibition of p38MAPK blandly inhibits FGF2-stimulated cell proliferation and migration, while it does not affect VEGF-induced cell proliferation and migration (Zeng, Zhao et al. 2002, Liao, Feng et al. 2009). Inhibition of JNK1/2 prevents cell migration stimulated by VEGF. Activation of AKT1 is necessaire for VEGF- and FGF2-induced eNOS activation and NO generation (Zheng, Bird et al. 1999, Zeng, Zhao et al. 2002, Mata-Greenwood, Liao et al. 2008) and *in vitro* angiogenic responses such as cell proliferation and migration and tube formation (Zeng, Zhao et al. 2002, Liao, Feng et al. 2009). However, only FGF2 induces eNOS mRNA and protein expression through ERK1/2 activation and AP-1 dependent transcription in placental endothelial cells (Mata-Greenwood, Liao et al. 2008, Mata-Greenwood, Liao et al. 2010). This remarks that placental angiogenesis is regulated by an intricate network.

4.3 CRH family of peptides

4.3.1 **CRH**

4.3.1.1 Gene expression

CRH is a 41-amino acid peptide extracted from the hypothalamus of sheep. The gene encoding CRH in the human genome is positioned on the long arm of chromosome 8 (Arbiser, Morton et al. 1988). The human, and sheep and rat, *CRH* gene comprises of two exons that is separated by an intron in its 5'-untranslated region. This region may play a key role in the regulation of *CRH* gene (Shibahara, Morimoto et al. 1983, Roche, Crawford et al. 1988, Suzuki, Kawasaki et al. 2009) because of their alignment which reveals the highest homology (94%) in the 330-bp proximal segment of the 5'-flanking region of the genes. Essentially, there is complete conservation of several consensus regulatory elements, including a 1/2 estrogen response element (ERE), cAMP response element (CRE), human placental-specific regulator, an ecdysone regulatory element, and two TATA boxes (Scatena and Adler 1998).

CRH-synthesizing neurons are situated in the paraventricular (PVN) nucleus of the hypothalamus, and CRH is secreted into the hypophyseal portal blood system, which transports it to the anterior pituitary gland. Within the central nervous system, CRH has also been identified in many areas outside the PVN, including the central nucleus of the amygdala and limbic structures , prefrontal cortical area, locus coeruleus, the dorsal raphe, inferior oliva nucleus, and Barrington's nucleus, where CRH may function as a neurotransmitter or neuromodulator(Swanson, Sawchenko et al. 1987). The immunoreactivity of CRH has also been demonstrated in a number of tissues outside the central nervous system, including the pancreas, lungs, liver, adrenal, stomach, and small intestines (Suda, Tomori et al. 1984). In addition, CRH immunoreactivity has been shown in several reproductive tissues such as germ cells , Leydig cells, spermatozoa(Yoon, Sklar et al. 1988, Audhya, Hollander et al. 1989) , myometrium(Clifton, Telfer et al. 1998), ovary(Mastorakos, Webster et al. 1993) , placenta (Shibasaki, Odagiri et al. 1982) endometrium (Petraglia, Tabanelli et al. 1992) uterine cervix(Klimaviciute, Calciolari et al.

2006). Despite profound glucocorticoid deficiency, CRH-deficient mice exhibited normal postnatal growth, fertility, and longevity(Arbiser, Morton et al. 1988). The development of fetus however, was profoundly affected by this deficiency. Due to lung dysplasia, the progeny of homozygous CRH- deficient mice all died within the first 12 h after birth. Additionally, marked atrophy of the zona fasciculata of the adrenal gland, that is involved in corticosterone production in mice, was observed, although the pituitary gland appeared normal. These observations indicate that CRH plays a critical role during stress.

4.3.1.2 Placental localization and expression

CRH expression was localized in human placenta, 20 years ago; it is now known that CRH production in placenta is confined to anthropoid but not prosimian primates(Grino, Chrousos et al. 1987, Smith 2007). From an evolutionary point of view this is interesting, because the hypothalamic CRH/CRH receptor system is conserved across vertebrates extensively, emphasizing its critical role in species survival. What is even more fascinating is that other placental mammals, including the rodents, use the CRH/CRH receptor in a similar way as humans to regulate a number of diverse physiological mechanisms, such as HPA axis activation, neuroendocrine control of the cardiovascular and immune systems. During the evolution of the endocrine control of pregnancy in mammals, the placental CRH system may have been preserved (or developed *de novo*) in primates for unknown reasons to influence the physiological outcome of pregnancy. The mature CRH is produced as part of a prohormone that is enzymatically processed from placental tissues. It is similar to the hypothalamic CRH in immunoreactivity, structure, and bioactivity (Chan, Thomson et al. 1988, Sasaki, Tempst et al. 1988). immunoreactive CRH and CRH mRNA, and protein expression is higher in placenta at term than at early gestation., the reticular layer of the chorion, the decidual stromal cells, the subepithelial layer of amnion and human umbilical vein endothelial cells also produce CRH and CRHR2 mRNA. Additionally, CRH mRNA is expressed in the cervix of the uterus, but at low levels in corpus. Enhanced CRH expression is seen in the placenta than in the myometrium, which further leads to high maternal plasma CRH levels(Shibasaki, Odagiri et al. 1982, Petraglia, Sawchenko et al. 1987, Saijonmaa, Laatikainen et al. 1988, Sasaki, Tempst et al. 1988, Riley, Walton et al. 1991, Simoncini, Apa et al. 1999, Grammatopoulos 2008).

the P4 synthesis inhibitor trilostane and Antiprogestin RU486 have inducing effects on gene transcription of CRH in a dose-dependent manner, thus supporting that endogenous P4 has a tonic inhibitory effect on synthesis of placental CRH. (Ni, Hou et al. 2004).

4.3.1.3 Regulation of placental secretion

Some mechanisms influencing placental CRH release are similar to those modulating CRH in the medial hypothalamic eminence of the brain. PGs, neurotransmitters (norepinephrine, acetylcholine), and neuropeptides (angiotensin II, arginine vasopressin), and IL-1 all induce release of CRH from cultured placental cells *in vitro.* CRH synthesis in placenta is reduced by estrogen, progesterone (P4), and by nitric oxide *(Petraglia, Sutton et al. 1989, Petraglia, Garuti et al. 1990)* . glucocorticoid-mediated inhibition of CRH release inhibits inappropriate overactivity of the 'stress' axis at the hypothalamic level, which can cause homeostatic mechanisms derangement; thus resulting in development of various, neuroendocrine, psychiatric and neurological illnesses, such as c, melancholic and atypical depression, the metabolic syndrome, chronic anxiety disorder and gastrointestinal disease. However, Contrary to the hypothalamic CRH system, the placental CRH production is up-regulated positively by glucocorticoids(Robinson, Emanuel et al. 1988) . This positive feed-forward system is a distinctive characteristic of placental CRH, representing a different role in pregnancy. this further indicates that as pregnancy progresses toward term, such "safety switch-off" mechanisms are not required by CRH and that the necessity for CRH actions rises. CRH is regulated differentially by various molecules(Simoncini, Apa et al. 1999) including 17β-estradiol , forskolin, progesterone, prostaglandin 2α, interleukin-1β and endothelin-1, and are potent mediators of CRH release. Molecules like dexamethasone reduces release of CRH. CRH exerts potent vasoactive effects on feto–placental compartment. administration of prostaglandin for induction of labour enhances levels of bioactive CRH via an enhanced levels of maternal plasma CRH and decreased levels of circulating CRH-BP. The resulting prostaglandin's positive feedback effect on release of maternal CRH might contribute to active labour(Florio, Lowry et al. 2007) .

CRH levels are very low (around 15 pg/ml) or undetectable in the sera of nonpregnant women. Contrasting to other CRH-related peptides, as human pregnancy progresses towards term, the placental-derived CRH levels rise exponentially, thus indicating that CRH may act as a 'placental clock' regulating the length of pregnancy (Smith 2007). Within 24 h after delivery, the peptide is undetectable (Goland, Wardlaw et al. 1986, Economides, Linton et al. 1987). There is a mid-pregnancy peak in production of placental CRH in monkeys and marmosets. There is an exponential increase only in great apes (chimpanzees, and gorillas) similar to that in humans and great apes also synthesize a circulating binding protein for CRH (CRH-BP) (Petraglia, Potter et al. 1993). Towards the end of gestation, CRH concentrations reduce, thereby enhancing CRH's bioavailability. In humans, women destined to have premature labour have enhanced CRH concentrations during mid-pregnancy than those who deliver at term, CRH may be utilized as a marker for identifying women at risk of prematurity(McLean, Bisits et al. 1995) . CRH is also localized in cord sera and amniotic fluid (Maser-Gluth, Lorenz et al. 1987, Nagashima, Yagi et al. 1987). CRH in fetal plasma is almost exclusively of placental origin are is secreted at half of maternal plasma(Mastorakos and Ilias 2003) .

There is a substantial rise in maternal circulating concentrations of CRH, possibly suggesting adaptive 'stress-related' placental mechanisms activation in abnormal pregnancy states (e.g. in pre-eclampsia and intrauterine growth retardation), (PERKins, Linton et al. 1995) . In idiopathic preterm labour, CRH concentrations rises up to 10 weeks before the development of any symptoms. There is a correlation between maternal estradiol and CRH thus indicating that CRH might be involved in placental estrogen biosynthesis.

4.3.1.4 Functional diversity of CRH system

4.3.1.4.1 CRH and hormone secretion

Multiple fetal–maternal tissues, including placenta, myometrial smooth muscle, fetal adrenals, fetal membranes, and (Grammatopoulos 2007, Smith 2007) are targeted by CRH during pregnancy. CRH mediates different actions in the placenta and fetal membranes, including: 1) human trophoblast cell growth and invasion via regulation of carcinoembryonic antigen-related cell adhesion molecule-1; 2) tissue remodeling via modulation of matrix-degrading protease matrix metalloproteinase-9 (MMP-9) release; 3) placental vascular tone control via NO pathway activation; and 4) direct regulation of the, PG , ACTH synthesis, and bioavailability and endocrine function (Jones and Challis 1989, Clifton, Read et al. 1994, Li and Challis 2005, Bamberger, Minas et al. 2006, Gao, He et al. 2007, Grammatopoulos 2007). Antalarmin, CRHR1 antagonist reduces secretion of PG E2 (PGE2), while, the CRHR2 antagonist, astressin-2b, does not alter placental release of PGE2, suggesting that CRH and CRH-related peptides synthesized locally exert different effects on PG synthesis via primarily interacting with CRHR1 and not CRHR2. CRHR1 and CRHR2 displays distinct effects on PGs production and metabolism In placental and chorionic tissues, indicating that in intrauterine tissues, CRH related peptides effect on output of PG are dependent on context of cell (Gao, He et al. 2007).CRH induces inositol triphosphates synthesis (Karteris, Grammatopoulos et al. 2001), which may play as signal to biosynthesis of PG secondary to PGHS-2 induction in decidua , amnion, chorion, placenta, *in vitro*(Jones and Challis 1989, Jones and Challis 1990, McKeown and Challis 2003) .

4.3.1.5 CRH and immune function

The CRH system regulates these events including peripheral vascular changes, including enhanced angiogenesis and /or permeability, are a major component of the inflammatory process. For instance, as shown by paw edema assays, CRH related peptides initiated mast cell degranulation in skin and increased vascular permeability, (Saijonmaa, Laatikainen et al. 1988, Grammatopoulos 2008); (Riley, Walton et al. 1991).

during vascular inflammation, CRHR1 applied vasoprotective effects: CRHR1 blockade enhanced significantly the tumor necrosis factor-mediated vascular adhesion molecule-1 and E-selectin expression in human aortic endothelial cells(Simoncini, Apa et al. 1999). Moreover, because CRH/CRHR1 stimulated inflammatory and endogenous vascular growth, whereas UCN III/CRHR2 blocked these responses, it was established that both CRH receptors have divergent effects on intestinal angiogenesis (Lovejoy and Balment 1999).

CRH is thought to regulate embryo implantation. Implantation consists of several steps resulting in effective cross talk among invasive trophoblast cells, various types of immune and nonimmune cells, and maternal endometrium. an intrinsic network of locally acting peptides has been established to control the formation of the adhesive endometrium and the inflammatory-like process of blastocyst implantation, and the subsequent processes of trophoblast invasion and placentation(Anin, Vince et al. 2004, van den Brule, Berndt et al. 2005) (Runic, Lockwood et al. 1996, Uckan, Steele et al. 1997, Xerri, Devilard et al. 1997, Huppertz, Frank et al. 1998, Runic, Lockwood et al. 1998, Bossi and Griffiths 1999, Kauma, Bae-Jump et al. 1999, Payne, Smith et al. 1999, Chatzaki, Makrigiannakis et al. 2001, Abrahams, Straszewski-Chavez et al. 2004). In certain cell types, including trophoblasts, CRH modulates expression of FasL expression via CRHR1. Minas *et al*. observed that FasL expression in DL is associated with spontaneous abortion in humans, along with enhanced Fas expression, and apoptosis of EVT *in situ*(Dermitzaki, Tsatsanis et al. 2002, Minas, Jeschke et al. 2007). During early implantation, by stimulating apoptotic FasL expression on invasive extravillous trophoblast and maternal decidual cells at the fetal-maternal interface, CRF binding to CRF-R1 results in the physiological immune tolerance. Unbound CRF levels increase during pregnancy and that CRF enhances vasodilation of placenta and myometrial contractility.

Placenta-derived CRH has been thought to act key role in the regulating mechanisms for maintenance of pregnancy in humans. CRH inhibits EVT invasion *in vitro* by CRHR1 activation(Phillips, Ni et al. 1999, Hillhouse and Grammatopoulos 2006). This effect includes the down-regulation of carcinoembryonic antigen-related cell adhesion molecule 1 production by EVT. Antalarmin enhanced EVT invasion by approximately 60%. Thus, enhanced EVT invasion may result in placenta accreta, and on the other hand,

aberrant invasion of cytotrophoblast has been implied in preeclampsia, indicating that the aberrant regulation of the CRH/CRHR1 system may result in placenta accreta and preeclampsia. CRH is thought to play a key role in events of implantation, ranging from endometrial decidualization to blastocyst invasion and fetal immunoescape from maternalrejection in response to physiological and pathological conditions, At the maternal–fetal interface, trophoblast cells are key in synthesis of significant proinflammatory cytokines (Griesinger, Saleh et al. 2001, Abrahams and Mor 2005) CRH has been reported to be released in high levels at the inflammatory sites locally and has a proinflammatory function by activating the immune/inflammatory response (Zoumakis, Margioris et al. 2000, Agelaki, Tsatsanis et al. 2002, Makrigiannakis, Zoumakis et al. 2003). CRH regulates myometrial expression of proinflammatory cytokine during human pregnancy(Aggelidou, Hillhouse et al. 2002). In TLR family, TLR-4 is the major receptor that responds to LPS, and it is localized in trophoblast cells. Enhanced expression of TLR-4 is seen in CRH treated cultured HTR-8/SVneo cells, indicating that the trophoblast's sensitivity to LPS may be modulated by CRH at the level of TLR-4 expression(Guleria and Pollard 2000, Aggelidou, Hillhouse et al. 2002, Holmlund, Cebers et al. 2002, Kumazaki, Nakayama et al. 2004, Tsatsanis, Androulidaki et al. 2006) .

4.3.1.5.1 CRH in Cardiovascular Function

Administration of CRH or UCN I Systemically might be in opposition of the CNS effects and thus reduce blood pressure by enhancing vasodilation in specific vascular beds (Goland, Wardlaw et al. 1986). Moreover, UCNs were observed to have a protective effect in heart failure and myocardial infarction, possibly via CRHR2 activation.42 cardiomyocytes were protected by UCN II and UCN III from hypoxia and infarct size decreased by anti- apoptotic ERK1/2 pathway activation(Economides, Linton et al. 1987, Maser-Gluth, Lorenz et al. 1987). In comparison to wild-type mice , CRHR2-null mice showed increased mean arterial pressure and diastolic pressure, indicating that changes in blood pressure and cardiac function are critically dependent on CRHR2.45 (Suda, Tomori et al. 1984, Petraglia, Sutton et al. 1989). The mechanism by which the CRH system controls vasodilation is not yet clear. Vasodilation may be regulated by endothelial NO release and the subsequent activation of the guanylyl cyclase signaling

pathway.46 Alternatively, endothelium-independent relaxation may occur via cAMP-dependent protein kinase A activation, which decreases the calcium sensitivity of contraction(Smith 2007).

4.3.1.5.2 CRH and vascular tonus

CRH is an effective, potent vasoactive molecule. Binding sites for CRH are localized on human vascular endothelium as well as smooth muscle. CRHR2 subunits, specially the isoform CRH2β, are localized in the vasculature(Dashwood, Andrews et al. 1987, Rohde, Furkert et al. 1996) . CRH causes vasodilatation of various vascular beds (Lei, Richter et al. 1993, Jain, Vedernikov et al. 1999, Harville, Savitz et al. 2008). Chronic administration of CRH in pregnant rats reduces blood pressure (Jain, Shi et al. 1998). Furthermore, CRH-induced vasorelaxation is a receptor-mediated, specific, endothelium-dependent effect via nitric oxide-cGMP pathway as well as endothelial-derived hyperpolarizing factor (epoxy eicosatrienoic acid), but not prostacyclin(Clifton, Read et al. 1995, Jain, Shi et al. 1998). CRH's vasoactive properties may be of great importance during human pregnancy physiologically, and the fetoplacental circulation is a direct target for CRH.

CRH perfusion of human placental lobules perfusion with CRH initiates a potent vasodilatation, that is mediated via a nitric oxide-cGMP pathway,(Clifton, Read et al. 1995). Furthermore, significance of CRH in the regulation of human fetoplacental circulation are given indirectly by pregnancies complicated by preeclampsia and intrauterine growth retardation, which are supplemented with abnormal placental vascular resistance and elevated concentrations of umbilical vein CRH (Trudinger, Giles et al. 1985, Goland, Jozak et al. 1993) .

Umbilical vein endothelial cells are potential target and a source for CRH. In umbilical vein endothelial cells, CRH is observed to be positively regulated by cAMP and is induced by forskolin. CRH at the endothelial level may be possibly involved in the local regulation of the immune-inflammatory processes (Karalis, Sano et al. 1991, Vamvakopoulos and Chrousos 1994). In vascular endothelium, the cross talk between CRH and cytokines may denote a novel mechanism for the local regulation of the immune-inflammatory responses mediated by this neuropeptide.

Downregulation of placental CRH-R expression also was observed in abnormal pregnancies, including pre-eclampsia(Karteris, Goumenou et al. 2003) . An accelerated release of CRH observed in pre-eclamptic pregnancies might act as a counter-regulatory mechanism to compensate for the decreased receptor expression and signaling efficiency. Moreover, due to their respective receptors' downregulation, several hormonal signals that control expression and activity of nitric oxide synthase (NOS) have weakened effects. These include other G-protein-coupled receptors (GPCRs), such as CRLR and RAMP1, CRH-R type 1 and type 2, The reduced expression may have important functional consequences, including dampening of CRH and UCNs actions on mRNA expression of eNOS, NOS activation and production of cGMP, leading to compromised responses and decreased feto–placental vasorelaxation from pre-eclamptic placentas(Vatish, Randeva et al. 2006).

4.3.1.5.3 CRH and delivery

CRH (and possibly CRH-related peptides) appear to target multiple feto–maternal tissues, including the fetal adrenals, placenta and fetal membranes and myometrial smooth muscle during human pregnancy (Grammatopoulos 2007, Smith 2007). During the second trimester, Enhanced concentrations of placental CRH modulates the fetal pituitary-adrenal axis, that might be mature (Mastorakos and Ilias 2003). This causes enhanced fetal adrenal steroids output and would lead to maturation of fetal lungs in a synchronized mode of action with forces that prompt the labour onset. This occurs via direct fetal adrenal CRH-R1 activation by CRH (and UCNI), which results in downstream cortisol and DHEAS increased levels that can be utilized for placental estrogen synthesis. This action might be further augmented via expression of CRH-induced ACTH receptor, that may increase responsiveness of fetal adrenal to circulating ACTH and contribute to the late gestational increase in release of cortisol from fetal adrenals. Thus intracellular signals cascade causing pre-term labour might be initiated by maternal stress(Wadhwa, Culhane et al. 2001, Sirianni, Mayhew et al. 2005, Rehman, Sirianni et al. 2007).

CRH targets the myometrial smooth muscle in humans; thus supporting CRH's role in regulation of myometrial contractility/quiescence during pregnancy and labour. During pregnancy and labour, the myometrium expresses functional CRH-R (both type 1 and type 2) whose expression and activity appears to be regulated dynamically. Their role is not clearly stated, however their presence in the myometrium is linked to the pattern of release of placental CRH, which peaks at term and the labour onset, (Keller, Kirkwood et al. 2003) . *in vitro* studies however indicates the possible role of CRH is thought to avoid premature myometrial contractions, increases myometrial contractions at term to facilitate pregnancy to reach term to enhance survival chances of fetus(Grammatopoulos 2007, Smith 2007). labour is linked with the two splicing events induction modulating myometrial CRH-R1 variants expression by deleting sequentially exons 6 and 13(Grammatopoulos and Hillhouse 1999) to produce CRH-R1α and CRH-R1d mRNA respectively, a process that reduces expression of mRNA of the 'pro-CRH-R1' receptor variant, CRH-R1β.

Distinct functional roles of CRHRs appear to exist in the control of myometrial contractility. recent isometric contractility and mathematical modeling studies in myometrial tissue strips provides evidence that The R1 subtype activate intracellular signals in the myometrium such as cAMP, cGMP and NO(Grammatopoulos, Stirrat et al. 1996, Grammatopoulos and Hillhouse 1999, Aggelidou, Hillhouse et al. 2002) that further reduces key contractile protein myosin light chain (MLC20) phosphorylation, and thus facilitating smooth muscle relaxation resulting to a quiescence state of myometrium. This is reduced with the labour onset due to reduced expression levels of key signaling proteins, such as Gsα-protein, thus shifting the balance of intracellular microenvironment towards myometrial contractility. Contrary to this, R2 activation induces signaling pathways, such as the RhoA-MAPK pathway, which enhances MLC20 phosphorylation and myometrial contractility. CRH-R2 is one of the genes in fundus that is significantly elevated during labour(Europe-Finner, Phaneuf et al. 1994, Karteris, Hillhouse et al. 2004, Mignot, Paris et al. 2005, Bukowski, Hankins et al. 2006). The full spectrum of the role of CRH-R2 in myometrium has not yet been elucidated. Other than signaling pathways modification, myometrial CRHR2 exert transcriptional effects since it can activate molecules including ERK1/2 and direct them to the nucleus to regulate as yet unidentified, specific genes activity.

Either at term or pre-term, myometrial CRH-R1s, localized in the lower segment of the uterus, is up-regulated with labour onset (Markovic, Vatish et al. 2007). It is likely that the CRH-R1 in myometrium exerts a multifaceted role and can cause diverse biological responses during different stages of gestation and labour. Indeed, its ability to and activate and couple multiple G-proteins (and subsequently signaling cascades) allows such diverse functions. The circulating CRF levels progressively rises during pregnancy. At the end of pregnancy, CRF-binding protein concentrations fall dramatically, so free CRF levels rises: this leads to the induction of uterine smooth muscle contractions and labor.

4.3.1.5.4 CRH and preterm labor

In the presence or absence of amniotic cavity's microbial invasion, preterm labor leading to preterm delivery was related with enhanced concentrations of CRH as well as lower CRHBP in both umbilical cord and maternal plasma than term pregnancies. Nevertheless, preterm labor with uterine cavity microbial invasion was correlated with significantly elevated plasma CRH concentrations than those seen in preterm labor without microbial invasion (Tropper, Warren et al. 1992, Warren, Patrick et al. 1992, PERKins, Eben et al. 1993, Petraglia, Aguzzoli et al. 1995, BERKowitz, Lapinski et al. 1996, Florio, Woods et al. 1997, Nodwell, Carmichael et al. 1999).

mRNA expression of CRH, but not CRHBP, is elevated in both term and preterm labor (PERKins, Eben et al. 1993, Florio, Woods et al. 1997, Petraglia, Florio et al. 1997, Nodwell, Carmichael et al. 1999, Gibb and Challis 2002, Menon, Arora et al. 2008). The enhanced MRNA expression of CRH observed in placenta is in support with data reporting elevated maternal and fetal plasma CRH levels during term and preterm labor, thus indicating placenta to be a main source of CRH during labor(McLean and Smith 1999) . Decreased CRHBP concentrations seen in the maternal circulation than in fetal circulation suggest that CRHBP arises from fetal source and, accordingly, that changes in cord plasma CRHBP are associated with the events of parturition(PERKins, Linton et al. 1995, BERKowitz, Lapinski et al. 1996, Florio, Woods et al. 1997).McLean *et al.* demonstrated that second trimester CRH concentrations were significantly elevated in women who subsequently delivered preterm and were reduced in women who progressed post term. The presence

of a “placental clock,” involving CRH that was active from the early stages of gestation was speculated, which determines the duration of pregnancy and the timing of labor. maternal serum CRH levels has been thought to be a predictive marker to the early recognition of patients at risk of preterm birth. (BERKowitz, Lapinski et al. 1996) . (Korebrits, Ramirez et al. 1998) essentially it was observed CRH could be used as a predictor of preterm labor only in patients after 30 wk gestation. Before 28 wk pregnancy, CRH was not useful when infection rather than activation of fetal or maternal HPA is the main driver to preterm birth. (Smith, Smith et al. 2009) reported maternal plasma CRH percentage-daily-change was elevated in preterm than term. It was correlated positively with the P/E3 and E3/E2 ratios and E3 surge, steroid hormone changes that are linked to the labor onset. The combination of mid trimester levels of CRF with α-feto protein and a clinical risk factor score increases all single markers sensitivity without compromising specificity (McLean, Bisits et al. 1999, Sandman, Glynn et al. 2006). Based on the maternal CRH concentrations, the critical period for the early prediction of preterm delivery related to the time interval between 26 and 31wk. Nevertheless, in general, due to poor predictive values and low sensitivity of a single CRH measurement, there is little support for the clinical use of plasma CRH alone as a predictor of preterm delivery(Inder, Prickett et al. 2001) .

The CRH system modulates gastrointestinal tract stress-related changes. Subsequently, it has been associated as a therapeutic target for IBS treatment. When CRH related peptides are administered centrally, it mimics the stress-induced response in colon: (1) by activating CRHR2, CRH, UCN I, and UCN II delays gastric emptying and inhibits propagative contractions(Taché and Bonaz 2007).(Lenz, Burlage et al. 1988). (2) CRH and UCN I reduces transit and motility of small intestine but they inhibit gastric transit to a greater extent because autonomic control is more prominent in the stomach than small intestine (Lenz, Raedler et al. 1988, Stengel and Taché 2010). (3) CRH and UCN I via CRHR1 activation, independent of HPA axis activation, increase colonic motility2,3. CRH and UCN I, when administered peripherally, via CRHR2 activation, inhibits gastric emptying, and via CRHR1 activation, increases colonic motility and delays small intestinal transit(Martínez, Wang et al. 2002).

CRH family mediating signaling pathways and Cellular responses may also affect intestinal inflammation. While CRHR1 Activation enhances dextran sodium sulfate-induced colitis, CRHR2 activation decreased it(Im, Rhee et al. 2010). (Anton, Gay et al. 2004, Kokkotou, Torres et al. 2006).CRH when centrally administered, decreased inflammation in trinitrobenzenesulfonic acid-induced colitis(Million, Taché et al. 1999).

4.3.1.5.5 CRH in Angiogenesis

As a critical pathological component of gastrointestinal diseases, including IBD and colon cancer, angiogenesis promotes the progression of the disease by providing essential nutrients and by facilitating the immune cells recruitment and the release of chemokines, and cytokines, and matrix-degrading enzymes(Jackson, Seed et al. 1997). Regulators of angiogenesis, including basic fibroblast growth factor, vascular endothelial growth factor (VEGF), and interleukin-8, are potential targets for the treatment of several diseases related to vasculature.

Current evidence indicates that the CRH family is a novel regulator of angiogenesis in the endogenous vasculature development and in tumor and inflammatory angiogenesis and is thought to be a potential therapeutic target for vascular disease, which includes the treatment of chronic inflammatory diseases and cancer. In contrast to conventional angiogenic factors such as basic fibroblast growth factor and VEGF, the CRH family does not cause angiogenesis in all settings. Similar to angiopoietin-1, a potent angiogenic factor in the skin, the CRH family does not promote corneal neovascularization, thus indicating that the CRH family specifically targets certain vascular beds(Suri, McClain et al. 1998). A recent preclinical study showed that synthetic CRH, corticorelin acetate (CrA) when administered as a single agent, significantly delayed growth of tumor possibly by inhibiting tumor angiogenesis(Gamez, Ryan et al. 2011). Furthermore, when CrA administered along with bevacizumab, an anti-VEGF antibody, improved therapeutic outcomes were seen.

Given that on-going pre-clinical studies hint toward the use of the CRH family members as anti-angiogenic agents, additional studies are recommended to determine the mechanistic basis for novel therapeutic approaches for vascular diseases. The probable role of the CRH family in angiogenesis is further discussed. The endogenous role of the

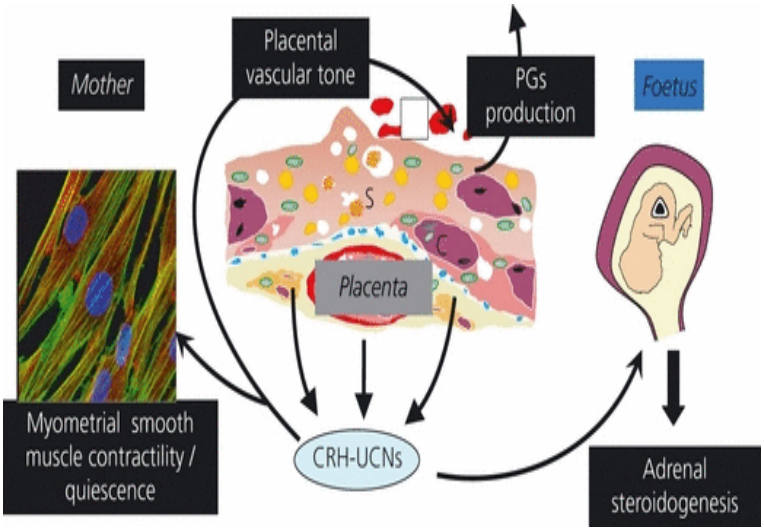
CRH system in development of vasculature was first examined by Bale et al.28 hypervascularization in CRHR2-deficient mice by postnatal day 21 suggested that CRHR2 inhibited postnatal neovascularization(Bale, Giordano et al. 2002).

The underlying mechanisms included inhibition of proliferation of smooth muscle cell (SMC), decreased release of VEGF from SMCs, and inhibition of angiogenesis in endothelial cells in vitro. Moreover, hypervascularity in CRHR2-deficient mice affected both capillaries and large vessels. Also, hypervascularization failed to occur during development but progressed from birth into adulthood. CRHR2 could affect the development of vasculature via two mechanisms: nitric oxide (NO) production and hypoxia(Bale, Giordano et al. 2003). NO regulates angiogenesis by increasing endothelial cell survival (Tsurumi, Murohara et al. 1997). CRHR2-induced NO production might affect local VEGF concentration, resulting in formation of vasculature. Hypoxia via expression of hypoxia inducible factor-1α, increases synthesis of VEGF. In hypertensive CRHR2-deficient mice, vasoconstricted vessels caused tissue hypoxia, which in turn leads to production of hypoxia inducible factor- 1α and VEGF.

4.3.1.5.6 CRH in Tumor Angiogenesis

CRHR2 is thought to be an endogenous inhibitor of angiogenesis and that antagonism of CRHR2/UCN might facilitate tumorigenesis. In a mouse xenograft model, CRH-overexpressing epithelial tumor cells enhanced angiogenesis and tumor growth by inducing endothelial chemotaxis, without an apparent effect on migration of smooth muscle cells(Arbiser, Karalis et al. 1999) . UCN reduced the tumor angiogenesis via the activation of CRHR2 and thus suppressed the tumor growths(Wang, Xu et al. 2008).

CRH belongs to a family of mammalian ‘stress’ peptides, including the urocortins (UCNs-UCNI, UCNII and UCNIII), which exert actions that are complementary or contrasting to fine-tune the actions of CRH. CRH is identified as a hypothalamic neurohormone regulating release of ACTH from the anterior pituitary lobe, which in turn induces release of cortisol from the adrenal cortex. Thus, CRH is a major regulator of several stress-induced behavioral, visceral and autonomic changes.



Grammatopoulos D.K. Journal of Neuroendocrinology 2009

Figure 3

CRH actions in pregnancy (adapted from Grammatopoulos D.K. Journal of Neuroendocrinology 2009)

4.3.2 **UCN**

4.3.2.1 Gene expression

Three CRH-like paralogs in mammals have been recognized since the CRH isolation: UCN I, UCN II (stresscopin-related peptide), and UCN III (stresscopin)(Vaughan, Donaldson et al. 1995, Hsu and Hsueh 2001, Vitoratos, Papatheodorou et al. 2006). a 40-amino acid peptide, UCN I, isolated from the rat midbrain, and UCN II and III, two 38-amino acid peptides, shares 45%−18% homology, respectively, with rat/human CRH (Imperatore, Florio et al. 2006). UCN shares a similar bioactivity and primary structure to both CRH and urotensin 1 (Skelton, Owens et al. 2000). The *UCN* genes for various species, including human, have been predicted or cloned and reported to be similar across species (Donaldson, Sutton et al. 1996, Zhao, Donaldson et al. 1998, Boorse, Crespi et al. 2005).The human *UCN* gene is located on chromosome 2 and codes for a 122-residue preprotein (2p23-p21). Similar to the *CRH* gene , It has two exons, with the coding region located in the second exon,(Donaldson, Sutton et al. 1996). By sequence homology, several possible transcription factor-binding sites have been identified in the promoter of UCN. Accepted regulatory elements include a a CCAAT enhancer-binding protein transcription factor-binding site, GATA-binding sites, , a binding site for the POU (Pit Oct

Unc) domain transcription factor Brn-2, TATA box, and a CRE (Zhao, Donaldson et al. 1998).

UCN has high affinity for CRHR1 and CRHR2 families and the CRH-binding protein (CRHBP) (Behan, Khongsaly et al. 1996, Lewis, Li et al. 2001). despite both CRHR1 and CRHR2 are activated by UCN2, the absence of a pervasive UCN projection to CRHR2-expressing cells and the lack of CRH/UCN projections to brain anxiety centers suggested the presence of additional CRH-related peptides(Bittencourt, Vaughan et al. 1999, Weninger, Dunn et al. 1999). At the N terminus, the predicted structures of mature UCN2 and UCN3 are different from those of other family peptides, despite CRH/UCN family peptides share similar secondary structures. Similar structure is shared by the mature peptides of CRH, UCN, urotensin I, and sauvagine with an N-terminal random coil followed by an extended α-helix structure.

Human UCN1 mRNA is expressed in various central nervous system cells and various peripheral tissues including gastrointestinal tract, heart, placenta, decidua, endometrium, fetal membranes. CRHRs are expressed in the human cardiovascular system. UCN2 and UCN3 is distributed in the heart, and causes sustained, direct and potent endothelium-independent vasodilating effects in an in vitro human internal mammary artery model of endothelin-1 stimulated constrictions and protects the heart against ischemic injury. UCN acts on calcium channels directly without binding to CRHR2. It decreases L-type and T-type calcium currents of acute isolated cardiac myocytes and blocks vascular smooth muscle cells' calcium channels. In other studies, UCN through increased ATP-sensitive potassium channels expression(Tao and Li 2005) produced endothelium-dependent vasodilating effects. Enhanced UCN concentrations are associated with the decrease in blood pressure and appetite via an increase in cardiac activity , contractility, and anxiety (Petraglia, Florio et al. 1996, Hsu and Hsueh 2001, Kalantaridou, Makrigiannakis et al. 2003). In the pancreatic alpha and beta cells, UCN3 was expressed and secreted during embryonic development in mice.

Endogenous UCN does not mediate in the HPA axis-mediated stress response. in the elevated plus maze and open-field tests, increased anxiety- like behaviors was shown in Urocortin-deficient mice; but their acute restraint-mediated response to stress did not differ from those in wild-type controls. UCN regulates anxiety via CRHR2. This is further

supported with a study which observed UCN null mice displaying the enhanced anxiety-like behavior, which was due to reduced CRHR2 MRNA levels in the lateral septum. Despite induction of a stress response and suppression of food intake by UCN administration, endogenous UCN deficiency did not alter the basal feeding behavior. Moreover, UCN is expressed in central auditory pathways, where it regulates auditory function. Accordingly, hearing was defective in UCN-deficient mice(Bittencourt, Vaughan et al. 1999, Vetter, Li et al. 2002).

4.3.2.2 Placental localization and expression

UCN is widely present in heart , small intestine and colon, immunological tissue, adipose tissue, skin, as well as syncytiotrophoblast, but is rarely found within mesenchymal cells of placental villi or cytotrophoblast(Muramatsu, Fukushima et al. 2000, Slominski and Wortsman 2000, Uzuki, Sasano et al. 2001, Kimura, Takahashi et al. 2002). UCN2 and UCN3 are widely distributed in muscle, colon, small intestine, peripheral blood cells, stomach, thyroid, adrenal, heart pancreas, brain, adrenal, and spleen and placenta. Although UCN2 and UCN3 are expressed in extravillous trophoblast (EVT) cells and syncytiotrophoblast, UCN2 is expressed to endothelial cells of blood vessels, thus indicating UCN2 to play a role in placental vascular endothelial cells. With respect to the fetal membranes, UCN and CRH are both expressed in amnion and chorion. While UCN2 is expressed only in amnion, UCN3 is seen in both amnion and chorionic cells (Imperatore, Florio et al. 2006).

4.3.2.3 Regulation of placental secretion

UCN regulates stress-related behavior and neuroendocrine activity and is expressed in paraventricular, arcuate, supraoptic, nuclei in the hypothalamus (Ikeda, Tojo et al. 1998). in human gestational trophoblasts *in vitro*, mRNA expression and release of UCN are not influenced by hypoxia, nevertheless UCN production was upregulated in hypoxic cardiomyocytes(Reyes, Lewis et al. 2001).

Not many studies are available on placental UCN2 and UCN3 regulation. UCN2 regulates fetal adrenal C19 steroid precursors conversion into E2 in placenta and this occurs by upregulating expression of P450 aromatase through CRHR2. CRH has been reported to directly enhance fetal adrenal C19 estrogen precursors output. Thus, CRH family peptides

is thought to have a role in the estrogen biosynthesis, eventually in physiological events of pregnancy and parturition (Imperatore, Li et al. 2009). In first trimester and in term trophoblasts, UCN expression is upregulated in a hypoxic environment via a hypoxia-inducible factor 1α-dependent manner. On the other hand, hypoxia decreases enzyme 11b-hydroxysteroid dehydrogenase type 2 expression, through which cortisol is inactivated by conversion to cortisone. Thus, in hypoxia with little amounts of cortisol inactivated, upregulation of CRH and UCN is observed within the placenta, potentially causing the pathway to increase estrogen biosynthesis reduced tumor growth by inhibiting angiogenesis via CRHR2 activation in cancers including renal cell carcinoma, lung carcinoma, hepatocellular carcinoma, prostate carcinoma.

4.3.2.4 Biological effects of UCN

UCN is not likely a physiological HPA axis regulator. Normal basal and stress-induced HPA hormone levels were displayed in UCN-deficient mice (Venihaki and Majzoub 2002)(318). at the hypothalamic level , UCN2 and UCN3 regulate activity of HPA-axis in a paracrine or autocrine fashion; indeed, they are enhanced in the parvocellular PVN after immobilization/restraint stress (Tanaka, Makino et al. 2003, Venihaki, Sakihara et al. 2004)(319, 320). Expression of Hypothalamic UCN2 is upregulated by glucocorticoids (Hashimoto, Nishiyama et al. 2004, Chen, Zorrilla et al. 2006) (322). UCN2 mediates synthesis and release of catecholamines from the adrenal medulla. In PC12 cells, it mediates release of noradrenalin and phosphorylation of tyrosine hydroxylase via protein kinase A and protein kinase A-ERK1/2 pathways, respectively (Nemoto, Mano-Otagiri et al. 2005). PVN UCN injection in rats up-regulated plasma leptin concentration, concluding that leptin could mediate anorexigenic effects of UCN (Kotz, Wang et al. 2002). NPY treatment causes a rapid induction of neuronal UCN mRNA(Gaszner, Korosi et al. 2007). In this sense, it appears that UCN provides a negative feedback to counteract the stimulatory effect of NPY on feeding. Gonzalez-Rey et al suggested therapeutic effects of UCN in colonic mucosa of mice, where UCN decreases the local and systemic concentrations of a wide range of inflammatory mediators, including cytokines, and chemokines.

In placental explants at term, UCN induced activin A (a member of the TGFβ superfamily) and PGE2 secretion in a dose-dependent manner; CRH and UCN –stimulated release of

PGE2 were almost similar and were reduced by Astressin (Muramatsu, Sugino et al. 2001, Florio, Arcuri et al. 2002). Like CRH, UCN (not UCN2 and UCN3) causes uterine and placental vessels dilation and relaxation via NO/cGMP pathway, via CRHR2 (Hsu and Hsueh 2001, Imperatore, Florio et al. 2006). high levels of maternal plasma CRF levels and reduced CRF-R1 expression are seen in Pregnancies characterized by abnormal placental function (e.g., preeclampsia)(Riley, Walton et al. 1991, Simoncini, Apa et al. 1999, Grammatopoulos 2008). This is supported by a study which observed decreased response of cGMP to UCN perfusion in placental explants obtained from preeclamptic women (Karteris, Vatish et al. 2005). Similarly, during mid pregnancy, defective uterine artery circulation was in association with decreased circulating concentrations of UCN. Furthermore, both CRH and UCN (not UCN2 or 3) causes placental vasodilatation to maximize the *in vivo* placental secretion of PGs or ACTH (Clifton, Owens et al. 1995, Petraglia, Florio et al. 1999). This is supported by a study which observed that CRH and UCN reduces a PGE metabolizing enzyme, 15-hydroxyprostaglandin dehydrogenase gene and protein expression in placenta, an effect that is reversed by selective CRH-R2 antagonist astressin.

4.3.2.5 UCN and delivery

4.3.2.6 UCN and preterm labor

UCN is secreted predominantly from the fetoplacental circulation into the maternal compartment. This is supported by studies which observed UCN was increased significantly in fetal circulation than in maternal plasma. Similar concentrations of UCN was found in the umbilical cord vein, as seen in the artery but at significantly lower levels. The human myometrial status (quiescence or contractility) correlates to concentrations of maternal plasma UCN as observed by their rise in labor at preterm and term(Florio, Torricelli et al. 2005) and reduction in post term pregnancy in comparison to term pregnancy(Torricelli, Ignacchiti et al. 2006). The concentrations of UCN in maternal sera, umbilical cord artery are elevated in preterm and term laboring women than in those seen in elective cesarean section patients(Florio, Torricelli et al. 2005).

Although mRNA levels of CRH in placenta and CRH levels in maternal plasma, rises throughout pregnancy, the levels of UCN does not(Dale 1906, Frim, Emanuel et al. 1988, Florio, Severi et al. 2002). The variable functions of CRH/UCN may have evolved so as to protect the fetus against environmental stress. However, under certain circumstances, it is possible that maternal or fetal stress could play a key role in triggering an intracellular signals cascade leading to preterm labor. A dual role for CRH/UCN is to promote relaxation of myometrium during most of pregnancy but stimulate the myometrial contractile response at term. This model proposes central role of CRH/UCN in coordinating the smooth transition from a state of quiescence to one of contractility emphasizing the importance of placental and fetal influence on the duration of human pregnancy, leading to postulate the existence of a placental as well as fetal clock that, through the CRF/UCN release, may determine the length of human pregnancy.

mRNA expression of UCN was not seen to be different significantly in tissue samples obtained during pregnancy or after preterm, term labor, and elective cesarean section (Dale 1906). Early placenta expressed UCN2 and UCN3 mRNA as early as 6-9 wk pregnancy, which by 10–12 wk decline, then increases by late first trimester again. The increased expression of UCN correlates with a period of relative placental hypoxemia, and expression of placental UCN2 and UCN3 was markedly induced by decreased oxygen tension in a HIF1a-dependent manner. Increased placental expression of UCN from severe preeclamptic pregnancies is in accordance with these results and raises the possibility that in early pregnancy UCN peptides may be associated with aspects of trophoblast proliferation and, potentially, implantation.

In patients with threatened preterm labor who later experience preterm delivery, maternal plasma concentrations of UCN are elevated. Furthermore, compared with those who deliver later, concentrations of UCN in women with preterm delivery are elevated in those who deliver before 7 d from admission. These findings, together with the evidence *in vitro* reported that UCN initiates contractile response in myometrium (Petraglia, Florio et al. 1999), support the hypothesis that maternal plasma levels of UCN are associated with the labor mechanisms and that increase in maternal plasma concentrations of UCN are associated with preterm labor. The evidence that maternal plasma levels of UCN is produced mainly from fetal sources raises new questions on the

role executed by the cross talk between the maternal and fetus environment in maintaining quiescence of myometrium as well as in the labor onset. Maternal plasma concentrations of UCN are elevated in threatened preterm labor who delivered before 34 weeks of gestation. Regardless of the physiological basis, this provides a potential tool to add significant prognostic information for predicting preterm delivery among at-risk women (Florio, Torricelli et al. 2005). Moreover, during the premature rupture of membranes (pPROM) with chorioamnionitis , CRH and its receptors are highly expressed in placenta, indicating that expression of stress-related pathways is activated in placenta during infective processes(Petraglia, Sawchenko et al. 1987).

CRH concentrations are reduced, in comparison with term pregnancies out of labor, whereas those of UCN were unchanged in post term. Compared to those that remained undelivered, Enhanced UCN concentrations in pregnancies delivered within 12 h of labour induction would support the idea that the combined effects of both neuropeptides are relevant in predisposing a response of myometrium to labor inducers (*i.e*., PGs). It has been shown that concentrations of fetal UCN are higher, whereas those of CRH are considerably lower than those in the mother. Thus it is thought that CRH has a placental source and UCN is of fetal origin in maternal blood.

1.1.1.1.1 Myometrium

Human myometrium expresses myometrium(Clifton, Gu et al. 2000). During labor, High UCN expression is seen in the myometrium and fetal membranes. The addition of UCN does not itself stimulate significant changes of myometrial contractility, but triggers response of myometrial contractility to endometrial prostaglandin F2a and the effect is abolished completely by astressin (Petraglia, Florio et al. 1999, Aggelidou, Hillhouse et al. 2002). UCN activates different intracellular signaling pathways that contribute to the myometrial contractility activation(Aggelidou, Hillhouse et al. 2002). The key factor is p42/p44, MAPK, which has been suggested to be associated in the myometrial contractility regulation by uterotonins. In cultured human pregnant myocytes, UCN via CRHR1(α) and CRHR2(β) stimulates MAPK activity thus initiating the cascade of events leading to contractility (Nohara, Ohmichi et al. 1996, Ohmichi, Koike et al. 1997). During human pregnancy, UCN2 regulates myometrial contractility, via CRHR2, and the sequential PKC activation, leading to the phosphorylation of MLC20(Tropper, Warren et

al. 1992). In the culture medium of amniotic epithelial cells, chorionic trophoblast, and syncytiotrophoblast, UCN induces protein level of MMP-9 without change in tissue inhibitor of metalloproteinase-1(Li and Challis 2005). This effect is potentially at the posttranslational processing level, including protein synthesis and secretion. Similar to CRH, the UCN induces MMP-9 and may indicate a local role for these peptides in cervical ripening and tissue remodeling at the time of labor.

At term, maternal plasma CRF and UCN levels are correlated to the onset of labour and are lower than at the beginning of pregnancy. In contrast, peptide levels do not reduce in post-term pregnancies. UCN correlates with the spontaneous abortion, labor onset, and pre-eclampsia(Hsu and Hsueh 2001). During the time of medical induction of post term labor, higher concentrations of UCN predict strongly a shorter time to delivery(Clifton, Read et al. 1996). The fine- regulated neuropeptides expression therefore, is important in regulating the length of human gestation(Clifton, Telfer et al. 1998). Low amniotic fluid UCN concentrations at mid-term may be thought a sign of predisposition to preterm birth(Shibasaki, Odagiri et al. 1982). Preterm pregnancy may be in association with a reduced vascular resistance in the fetal cerebral circulation(Sherer 1998, Florio, Torricelli et al. 2006). Fetal UCN secretion correlates with perfusion of uteroplacental bed(Florio, Torricelli et al. 2006), and that UCN has sustained protective and hemodynamic compensatory effects against hypoxia in animals. Thus it is suggested that secretion of UCN may protect the fetus against hypoxic insults (Rademaker, Charles et al. 2002, Fan, Chen et al. 2009). UCN, via CRH-R2, also up-regulated myometrial proinflammatory cytokines expression. Between UCN and inflammatory cytokines, A positive feedback loop therefore probably exists because expression of UCN was increased by the inflammatory stimulus tumor necrosis factor alpha, via NF-kB signaling(Arbiser, Morton et al. 1988).

4.3.2.6.1 Adrenals

UCN2 and UCN3 are differentially expressed in Gestational tissues and don't induce placental ACTH secretion(Imperatore, Florio et al. 2006). CRH, UCN, via CRHR1 induces human fetal adrenal glands DHEAS, which acts as a substrate for placental estrogen synthesis, further resulting in important changes during childbirth(Sirianni, Mayhew et al. 2005). Estrogens directly regulates transcription of UCN(Haeger, Andres et al. 2006).

UCN plays a role in the tumorigenesis of different tissues(Florio, De Falco et al. 2006) and ovarian steroidogenesis. UCN1 is expressed in both theca and granulosa cells of atretic follicles, dominant and non-dominant follicles. During the mid- and late-phase corpus luteum , UCN1 was also expressed in luteinized granulosa and thecal cells(Gonzalez-Rey, FeRNAndez-Martin et al. 2006). Maternal serum concentrations of UCN are elevated in women with endometriomas, thus to distinguish between other benign ovarian cysts and endometriosis.

4.3.2.6.2 Ovary

Immunohistochemistry methods also revealed that regressing corpus luteum expressed significantly higher MRNA levels of CRH and CRHR1 compared to either the mid-luteal phase or pregnant corpus luteum[21] Nevertheless, the expression of these genes in the mid-luteal phase of the menstrual cycle was not significantly different than early pregnancy(Gonzalez-Rey, Fernandez-Martin et al. 2006). During the menstrual cycle, mRNA of UCN has been observed to be expressed in endometrial stromal and epithelial cells during both endometrial proliferative and secretory phases. High expression of UCN is seen during secretory phase(Torricelli, De Falco et al. 2007, Xu, Xu et al. 2007)

Although there is a similarity substantially in CRF and UCN actions regarding myometrial contractility and placental functions, similar relationships were not shown when examining the ability of the placenta to produce UCN(Petraglia, Florio et al. 1999). It has already been observed that the placenta produces sufficient quantities of CRF, but it has been reported that the placenta does not produce sign cant quantities of UCN (Glynn, Wolton et al. 1998). UCN binding to CRH-R1a activates both the Gs and Gq in human myometrium and human embryonic kidney via activation of pathways such as adenylyl cyclase/protein kinase A and the phospholipase C/protein kinase C and ERK1/2 (Papadopoulou, Chen et al. 2004).

4.3.3 **CRHR signalling characteristics**

Table 1:

CRHR signalling characteristics

(https://academic.oup.com/view-large/61436623)

	CRHR1	**CRHR2**
Chromosome	17q21.31	7q14.3
Peptide length (amino acids)	415–446	397–438
Splice variants	CRHR1A;CRHR1C,E,H;CRHR1B,F; CRHR1G,D	CRHR2A; CRHR2B; CRHR2C
Endogenous ligands	CRH, UCN	UCN, UCN2, UCN3
Peripheral distribution	Placenta, ovary, skin, adrenal gland, endometrial cells, mast cells	Placenta, blood vessels, heart, skeletal muscle, gastrointestinal tract

CRHRs belong to the “brain-gut” neuropeptide receptors superfamily, which include receptors for vasoactive intestinal peptide, calcitonin, adenylate cyclase- activating peptide, PTH, secretin, glucagon, and GH-releasing factor(Segre and Goldring 1993). CRHR1 and CRHR2 differentially bind to each of the CRH family members. CRH has high affinity only for CRHR1, UCN shows approximately the same affinity for both receptors, whereas UCN2 and UCN3 bind with high affinity only to the CRHR2 (Grigoriadis, Lovenberg et al. 1996, Aguilera, Nikodemova et al. 2004). Compared to CRH, UCN-I, UCN-II and UCN-III are more potent at binding and activating CRH-R2 receptors, and these peptides are hence thought to be the native agonists for the CRH-R2 receptor. Estrogen regulates CRHR expression.

CRHRs has seven-transmembrane helical domains. Both CRHRs share 70% identity at the amino acid level, and several variants are produced as a result of differential RNA splicing (Dieterich, Lehnert et al. 1997). The CRH-R1 variants are produced by many partial or

complete exon(s) insertions or deletions (Pisarchik and Slominski 2001, Teli, Markovic et al. 2008). The CRHR1α subtype has been cloned from several species including mouse and human (Perrin, Donaldson et al. 1993, Vita, Laurent et al. 1993, Tsai-Morris, Buczko et al. 1996). In all species, mRNAs of CRHR1α encode proteins of 415 amino acids that are 98% identical to one another. Another variant of CRHR1, CRHR1β, consists of an additional 29 amino acids inserted into the first intracellular loop and has been recognized in the human pituitary. An additional presence of 29 amino acids in CRHR1β impedes with the receptor's signal transduction properties and decreases adenylate cyclase coupling(Chen, Lewis et al. 1993). CRHR1C, another variant of the human CRHR1α, has a 40-amino acid deletion in the N-terminal domain of the receptor (Ross, Kostas et al. 1994). CRHR1δ, a splice variant of the CRHR1α, with a deletion of 14-amino acid within the seventh transmembrane domain has been identified in myometrium and fetal membranes. CRHR1δ binds with high affinity and specificity to human CRH (Kd = 3.3 ± 0.45 nM) and is coupled functionally to adenylate cyclase (Hillhouse and Grammatopoulos 2001).

CRH-R2 gene demonstrates a completely distinct splicing pattern compared to CRH-R1, probably relevant to its distinct role in mammalian physiology. All CRH-R2 variants are different only in their N-terminal extracellular domains. However, different N-termini do not alter various CRH-related peptides' agonist binding and signaling properties significantly. three known isoforms of CRHR2 are present : CRHR2α, CRHR2β, and CRHR2γ(Hillhouse and Grammatopoulos 2001). CRH-R2α mRNA variant, encoding for a soluble protein, might play a role as a circulating binding protein for UCNs. This sCRH-R2α diminishes cellular responses to CRH and UCN, thus being a biological modulator of CRH and CRH-related peptides. CRHR2α is 411 amino acids in length, shares homology to CRHR1 approximately around 71% (Lovenberg, Chalmers et al. 1995, Liaw, Lovenberg et al. 1996, Valdenaire, Giller et al. 1997, Catalano, Kyriakou et al. 2003). CRHR2β, The alternatively spliced form, is 431 amino acids in length and is distinct from CRHR2α where the first 34 amino acids in the N-terminal extracellular domain are replaced by 54 different amino acids (Ardati, Goetschy et al. 1999, Evans and Seasholtz 2009). Similarly, CRHR2γ, has an N terminus with no significant homology to the CRHR2α and CRHR2β subtypes and encodes for 397 amino acids and is found in human brain(Horlick, Sperle et al. 1997, Kostich, Chen et al. 1998).

Generally, “fight-or-flight” stress response is mediated by CRHR1 via the CRH-ACTH-glucocorticoid axis, whereas stress-coping responses is mediated by CRHR2 via UCN II (stresscopin) and UCN III (stresscopin-related peptide)(Hsu and Hsueh 2001). CRHR1-deficient mice exhibited a marked decrease in the size of the zona fasciculata region of the adrenal gland and thus had a low plasma concentration of corticosterone. (Smith, Aubry et al. 1998). Although basal ACTH secretion was normal in these mice, restraint stress did not increase circulating ACTH levels. Within 2 days after birth, homozygous mutant females’ progeny exhibited lung dysplasia with alveolar collapse and emphysema and died. Corticosterone treatment in utero rescued the phenotype. The mutant mice were less responsive to anxiogenic-like stimuli, indicating that CRHR1 is necessary for a normal anxiety response. CRHR2-deficient mice exhibited increase in anxiety-like behavior, and hypersensitivity to the HPA axis-mediated stress response(Bale, Contarino et al. 2000). A decreased appetite, increased anxiety, and thus reduced food intake in response to food deprivation stress was observed in mice, despite the normal food intake pattern and weight changes. In the mutant mice, Intravenous UCN infusion did not decrease the mean arterial pressure but the nitric oxide-mediated vasodilation was normal in the peripheral blood vessels. Thus, CRHR1 mutant mice is thought to show decreased anxiety-like behavior, and CRHR2 mutant mice show increased anxiety-like behavior.

4.3.3.1 Placental localization and expression

CRH-binding sites are mainly expressed by the syncytiotrophoblast sites in human placenta (Clifton, Owens et al. 1995, Hatzoglou, Margioris et al. 1996, Grammatopoulos, Dai et al. 1999). CRHR1α and CRHR1χ, CRHR1δ and CRHR2β expressed Human syncytiotrophoblast, amnion, and chorion, but not scattered cytotrophoblast cells within the chorionic villi (Grammatopoulos, Dai et al. 1999). Three CRHR subtypes: 1α, 1β, and 2β are expressed in nonpregnant human myometrium. Myometrium begins to express CRHR2α, as pregnancy progresses. At term, additionally, the 1c and 1d receptor subtypes are expressed in the myometrium(Ross, Kostas et al. 1994). It was shown that CRHRs to be expressed on resident macrophages ,endothelial cells, trophoblast, myometrium, decidua, and fetal membranes.(Wetzka, Sehringer et al. 2003).

Although a significant rise in accumulation of total inositol phosphate (IP3, IP2, IP) was observed in response to CRH (Carsten and Miller 1985). CRHRs do not couple to Gs and adenylate cyclase in human placenta and fetal membranes. In most tissues, CRHRs are preferentially coupled to phospholipase C and adenylate cyclase (Ulisse, Fabbri et al. 1990). The CRHR may be coupled to the alpha-subunit of Gq, which subsequently results in phospholipase C activation rather than Gs, thus eventually leading to adenylate cyclase activation in the fetoplacental unit (Karteris, Grammatopoulos et al. 2001). CRH stimulation of placental membranes increased Gq, Go, and Gz labeling, but not Gi and Gs, whereas CRH stimulation of fetal membranes increased Go and Gq labeling, but not Gi, Gs, and Gz(Karteris, Grammatopoulos et al. 2000) .

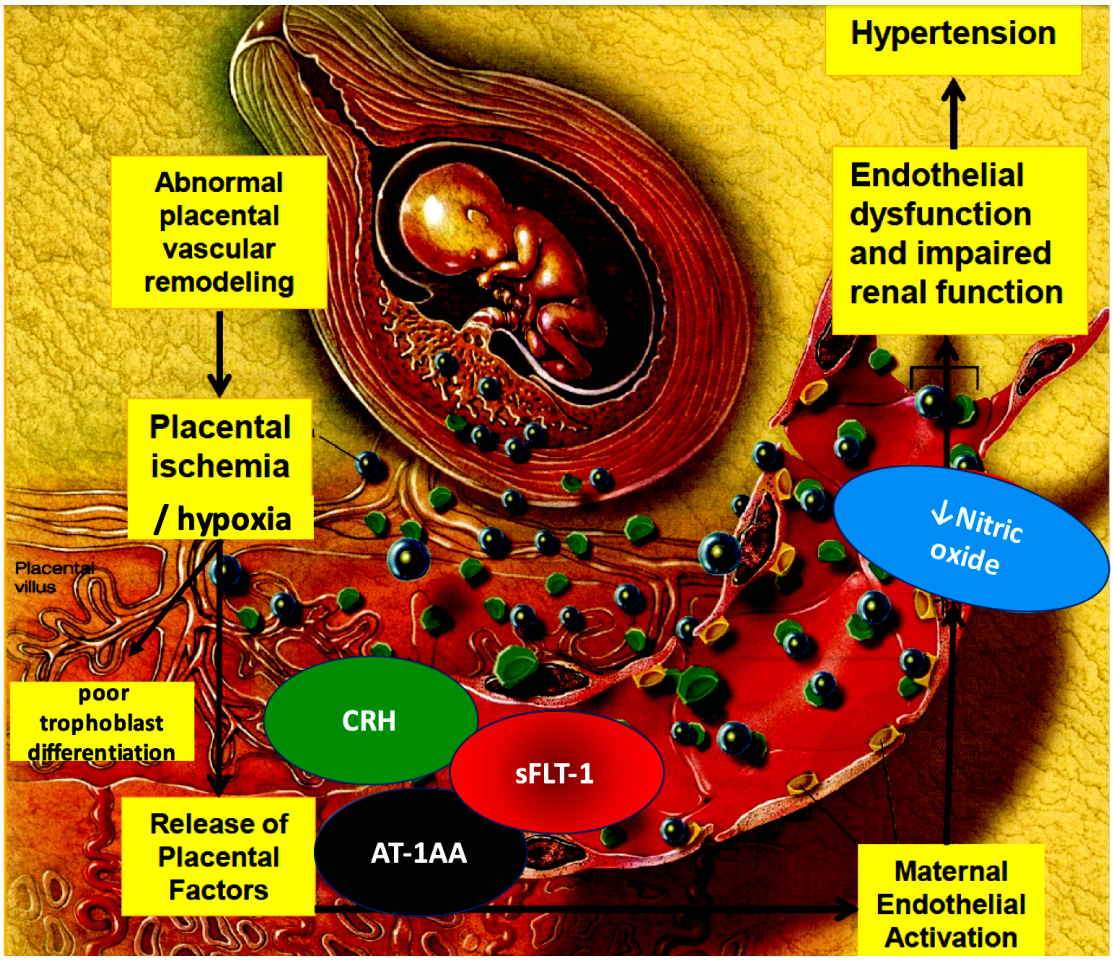


Figure 4

Stressed placenta leads to stressed mother.

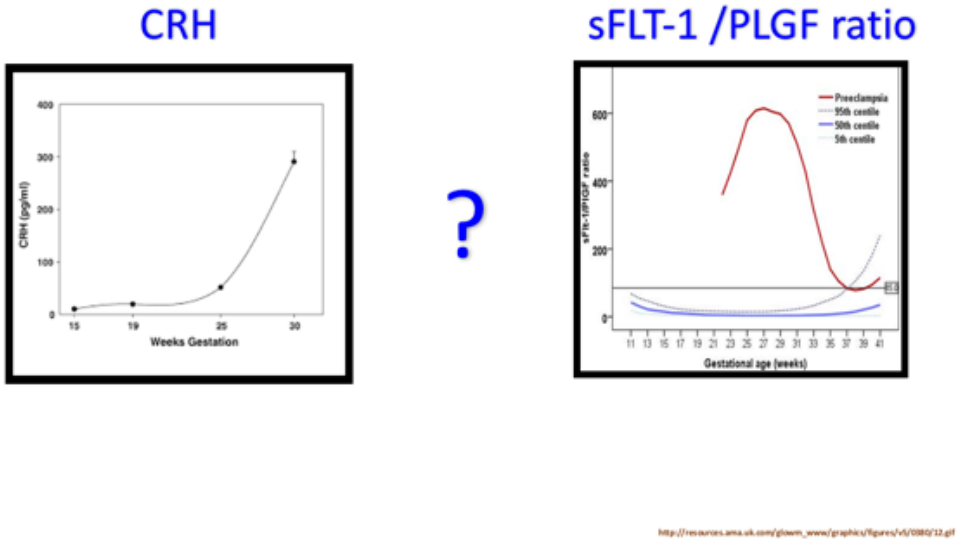


Figure 5

Unestablished relationship between CRH release and SFLT-1/PLGF ratio

4.4 Hypothesis

SFLT-1/PLGF ratio is recently proposed as one of the biomarkers that may be useful in the diagnosis and management of placental insufficiency syndromes such as preeclampsia. The placental CRH/UCN are suggested as important neuroendocrine mediators in the physiology of pregnancy and parturition(Petraglia, Imperatore et al. 2010). CRH family of peptides appear to be important regulator of angiogenesis in various human physiological systems. The reduces placental CRHR expression and elevated placental CRH secretion seen in preeclamptic women, may reflect an adaptation of the fetus in hypoxemic stress and provide a strong rationale for targeting CRH for protection against preeclampsia. Thus it is hypothesized that CRH regulates two important placental functions, trophoblast differentiation and angiogenesis in various gestational cellular models under altered oxygen tension representing placental related pregnancy disorders such as preeclampsia.

This study describes a series of analyses conducted with the aim to investigate the molecular mechanisms underlying placental dysregulation of angiogenic molecules leading to endothelial dysfunction in trophoblast and endothelial cells, and establish a potential link between their signaling pathways using in vitro models under altered oxygen tension. This project also explored the effect of SFLT-1 rich media on the fusogenic machinery and endocrine capacity of BEWO cells under altered oxygen tension.

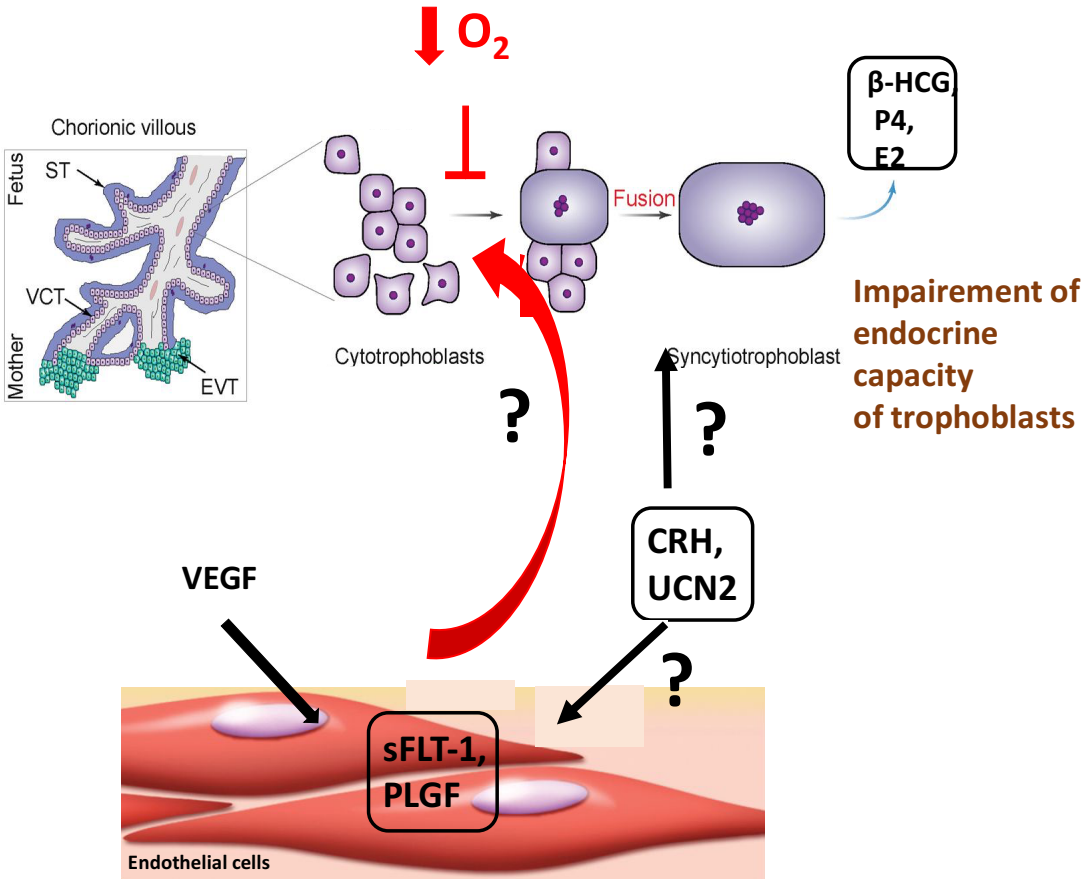


Figure 6

Diagrammatic representation of hypothesis (Gerbaud 2015) *https://www.frontiersin.org/articles/10.3389/fphar.2015.00202/full*

Research questions :

1. What is the role of CRH family of peptides on trophoblast differentiation under altered oxygen tension?
2. What is the role of CRH family of peptides on trophoblast invasion under altered oxygen tension?
3. What is the role of CRH family of peptides on placental angiogenesis under altered oxygen tension?

   Overall, can CRH family of peptides influence hypoxia mediated impairment of the functions of trophoblast and endothelial cells in placenta, so as to significantly alter the biological effects of trophoblast and endothelial cells?

To address question 1, BEWO cellular model was used, which were stimulated by CAMP activator, forskolin, so as to differentiate cytotrophoblasts to syncytiotrophoblasts. The cells were treated with CRH related peptides under 21% O2 and 3% O2. RNA, proteins of syncytins and B-HCG, Estradiol and Progesterone were quantified using QRT – PCR, Western blot and ELISA.

To address question 2, HTR8/SVneo cellular model was used. The cells were treated with CRH related peptides under 21% O2 and 3% O2. RNA, proteins of SFLT-1 and PLGF were quantified using QRT PCR, Western blot and ELISA.

To address question 3, HUVEC cellular model was used. The cells were treated with CRH related peptides under 21% O2 and 3% O2. RNA, proteins of SFLT-1 and PLGF were quantified using QRT PCR, Western blot and ELISA.

5 Materials and methods

5.1 Materials

Table 2:

List of chemical reagents

Chemical reagents	Product code	Manufacturer
Amersham ECL Prime Western Blotting Detection Reagent	12316992	Thermo Scientific Pierce
Bovine Serum Albumin (BSA)	29130	Gibco, UK
Carbenoxolone disodium salt	C4790	Sigma Aldrich, UK
Dimethyl Sulfoxide (DMSO)	10103483	Gibco, UK
Dulbecco's Modified Eagle Medium(DMEM)	11995-065	Gibco, UK
Ethanol	32221	Sigma Aldrich, UK
Fetal Bovine Serum (FBS)	41G5240K	Gibco, UK
Forskolin	3828S	Cell Signalling Technology, USA
Gentamycin / Amphotericin antibiotics (500X)	R-015-10	Gibco, UK
GenElute Mammalian Total RNA Miniprep Kit	RTN70	Sigma, UK
Glycine	G/0800/60	Fluka,UK
Ham's F-12K (Kaighn's) Media	21127-030	Gibco, UK
High capacity RNA-to-cDNA kit Applied Biosystems	Part Number: 4387406	Fisher Scientific, UK
Human corticotrophin releasing hormone (CRH) Acetate salt	H-2435	Bachem, Germany
Human Recombinant VEGF-165 (VEGF)	8065SC	Cell Signalling Technology, USA
Human Urocortin II Trifluroacetate salt (UCN2)	H-5852	Bachem, Germany
Hydrocortisone, 98%	10062031	Gibco, UK
Isopropanol	W292907	SAFC,UK
L Glutamine (100X), liquid 200mM	11534546	Gibco, UK
LSGS kit	S-003-10	Gibco, UK
MCDB 131 media	11513407	Gibco, UK
Media 200	M-200-500	Gibco, UK
2-Mercaptoethanol	161-0710	BioRad, UK
Methanol	322415	Sigma Aldrich, UK

OdysseyTM Blocking Buffer (TBS)	927-50000	LI-COR Biosciences UK Ltd. ®, UK
On-Column DNase I Digestion Set	DNASE10 and DNASE70	Sigma, UK
PageRuler(TM) Plus Pre-stained Protein Ladder	11832124	Thermo Scientific Pierce
Pierce phosphatase inhibitor mini tablets, EDTA-free	13424676	Thermo Scientific Pierce
Pierce protease inhibitor mini tablets, EDTA-free	13437766	Thermo Scientific Pierce
Pierce™ BCA Protein Assay Kit	23227	Thermo Scientific Pierce
Potassium chloride (KCl)	P9541	Sigma Aldrich, UK
Potassium phosphate monobasic (KH_2PO_4)	P0662	Sigma Aldrich, UK
5X Protein Loading Buffer	EC-887	National Diagnostics
30% Protogel	EC-890	National Diagnostics
ProtoGel Resolving Buffer (4X)	EC-892	National Diagnostics
ProtoGel Stacking Buffer	EC-893	National Diagnostics
Proteome Profiler Human Angiogenesis Array Kit	Ary007	R & D systems, UK
Radioimmunoprecipitation assay buffer (RIPA) lysis Buffer	10017003	Thermo Scientific Pierce
Recombinant Human Epidermal Growth Factor (EGF)	10533084	Gibco, UK
RPMI 1640 media	11875-093	Gibco, UK
Bioline SensiFAST SYBR Lo-ROX kit	BIO-94020	Bioline, UK
Sodium Chloride (NaCl)	S9625	Sigma Aldrich, UK
Sodium dodecyl sulfate (SDS)	S/P530/48	Fisher Scientific, UK
TEMED	T-9281	Sigma Aldrich, UK
TRIS base	BP152-1	Fluka, UK
Tris Glycine SDS Running buffer (10X)	EC-870	National Diagnostics
Tris-Glycine Electroblotting Buffer (10X)	EC-880	National Diagnostics
Trizma® hydrochloride (Tris HCl)	T-5941	Sigma Aldrich, UK
Trypan blue solution (0.4%)	15250-061	Gibco, UK
Trypsin EDTA solution (0.25%)	11538876	Fisher Scientific, UK
Tween 20	10485733	Gibco, UK
V-PLEX Human FLT-1 Kit	K151RZD-1	Meso Scale Discovery, USA
V-PLEX Human PLGF Kit	K151MED-1	Meso Scale Discovery, USA
V-PLEX Human VEGF Kit	K151RHD-1	Meso Scale Discovery, USA

Table 3:

List of buffers and solutions

Buffers and Solutions	**Components**
10% Ammonium persulphate (APS)	0.1g APS in 1ml double distilled water, made up prior to use and stored at 4^{o}C for a week
5% Acetic acid	5% (v/v) acetic acid in double distilled water
5% BSA in TBST	1x TBS, 0.1% Tween-20 with 5% (w/v) BSA
5% Milk (Blocking buffer)	1x TBS, with 5% (w/v) milk
5% Milk in TBST	1x TBS, 0.1% Tween-20 with 5% (w/v) milk
Phosphate Buffer Saline (PBS)	0.2g/L KCl, 0.2g/L KH_2PO_4 (anhydrous). 8.0g/L NaCl, 1.15g/L Na_2HPO_4 (anhydrous), pH 7.4
Ponceau -S staining solution	0.5% (w/v) Ponceau -S, 1% (v/v) acetic acid in double distilled water
10X SDS-PAGE (polyacrylamide gel electrophoresis) running buffer	30g Tris -HCl, 144g glycine, 10g SDS, double distilled water to 1000ml
Stripping buffer (ECL Detection System)	6ml of 10% SDS, 1.875 ml of 1M Tris - HCl (pH 6.8), 233 µl β mercaptoethanol, double distilled water upto 30ml
Stripping buffer (Odyssey Infrared Imaging System)	25mM glycine pH 2, 2% (w/v) SDS
10X Tris Buffer Saline (TBS)	24.2g Tris- base, 80g NaCl upto 1000ml double distilled water, pH 7.6
10X TBS tween (TBST)	24.2g Tris- base, 80g NaCl upto 1000ml double distilled water, pH 7.6, 0.1% Tween- 20
10X Transfer buffer	7.5g Tris HCl, 36g glycine, 500ml methanol, double distilled water to 2500ml

Table 4:

List of cellular models

Cells	**Company**
BEWO	American Type Culture Collection (ATCC), USA

HMEC-1	Centre for Disease Control, USA
HTR8/SVneo	Queen's University, Canada
HUVEC	Caltag Medsystems Ltd, UK

Table 5:

List of other consumables

Materials	**Product size/code**	**Manufacturer**
Cell scraper, small, Corning	3010	Appleton Woods
Chromatography paper Grade No. 3MM (filter paper, 0.34mm thick)	10441412	GE Healthcare Whatman
Cell culture flasks	25/75cm^2	Cellstar, UK
Cryovials	1.5ml	
Eppendorfs	1.5/0.5/0.2 ml	-
Falcon tubes	15/50ml	Greiner
Fibre pad	11857653	Gibco, UK
Cell culture plates	6 well	Corning Incorporated costar, UK
Mr Frosty-NALGENE® labware (Nalge Nunc)	5100-0001	Thermo Scientific, UK
Nitrocellulose membrane (Amersham(TM) Protran Premium 0.45µm)	10600036	GE Healthcare Life Science
Pipette tips	1000/200/100/20/10/1ul	
Serological pipettes	25/10/5ml	Greiner

Table 6:

List of instruments

Instruments	**Instrument details**	**Manufacturer**
Balance	PF-6001	Fisherbrand
Centrifuge	Centrifuge Micro Centaur	MSE
	Sorvall Legend RT	Thermo Scientific

	Sorvall Evolution RC	Thermo Scientific
	Rotor: Aluminum Fixed Angle, SS-34	
FAST 7500 qPCR machine	FAST 7500	Thermo Scientific
G:BOX Chemi XX6	GeneSys v1.4.1.0	Syngene, UK
Heat block	QBT4 Digital Block Heater	Grant
Haemocytometer		
MSD SECTOR ® Imager 6000	Version 3.0	Meso Scale Discovery (MSD), Gaithersburg, MD, USA
IKA® VXR basic Vibrax® orbital shakers		Sigma Aldrich, UK
pH meter	Orion ROSS Ultra pH/ATC Triode	Thermo Scientific
Pipette aid		Sartorius, UK
Pipettes	Pipetteman 1-10/10-100/30-300/100-1000 µL	Gilson, UK
Tecan microplate reader	MagellanV5.03	Tecan, Switzerland
Stirrer	stir, CB161	Stuart
Super-Q plus water system	Carbon filter Ion exchange Durapore 0.2 µM	Millipore
Vortex	VX100 Labnet	MO BIO Laboratories, Inc
Western blot apparatus	Mini Trans-Blot® cell	BioRad, UK
Odyssey Infrared imaging system	Version 3.0	LI-COR, UK

5.2 Methods

5.2.1 **Cell culture methods**

5.2.1.1 Cell cultures

5.2.1.1.1 Trophoblast cell lines

5.2.1.1.1.1 BEWO cell line

BEWO, a human choriocarcinoma cell line, is a well-established model for placental differentiation. It is maintained in an undifferentiated state that further differentiates in presence of forskolin(Wice, Menton et al. 1990, Lin, Xu et al. 1999).

5.2.1.1.1.2 HTR-8/SVneo cell line

HTR-8/SVneo cell line is often used to model the physiologically invasive extravillous trophoblasts (EVT) and was originally isolated from 1st trimester trophoblasts. HTR-8/SVneo cell line is a transformed EVT cell line that is established by immortalizing physiologic EVT cell via transfection with a plasmid containing the simian virus 40 large T antigen (SV40)(Graham, Hawley et al. 1993). It is obtained from Queen's University, Canada. This model will be used to study characteristic features of EVT such as proliferation, invasion and migration.

5.2.1.1.2 Endothelial cell lines

5.2.1.1.2.1 Human Umbilical Vein Endothelial cells (HUVECs)

HUVECs that are isolated from fetal umbilical cords and maintained in primary cell culture will be used to study endothelial cell function. The experiments will be performed between 3-5 passages as some of the key molecules of target, such as eNOS expression will decrease in higher passaged cells.

5.2.1.1.2.2 Human Microvascular Endothelial Cells (HMEC-1)

HMEC-1 is established by transfecting dermal human microvascular endothelial cells from human foreskin with a plasmid containing the simian virus 40A gene(Ades, Candal

et al. 1992). They are maintained in continuous cell culture. Their advantages are similar to that of any continuous cell line.

All cell culture work was carried in Class II; type A laminar flow culture hood assigned for handling animal tissues or human material, using aseptic precautions. The media for each cell line was prepared as per table below.

Table 7:

Media composition for each cell line

Cells	**Media**
BEWO	F12K Media with 10% FBS, 10 µg/ml gentamycin and 0.25 µg/ml amphotericin B
HUVEC	Media 200, Low Serum Growth Supplement kit (2% v/v FBS; 1 µg/ml hydrocortisone; 10 ng/ml human epidermal growth factor; 3 ng/ml basic fibroblast growth factor; 10 µg/ml heparin); 10 µg/ml gentamycin and 0.25 µg/ml amphotericin B
HTR8/SVneo	RPMI 1640 media, FBS to a final concentration of 5%, 10 µg/ml gentamycin and 0.25 µg/ml amphotericin B
HMEC-1	MCDB131, 10ng/mL Epidermal Growth Factor (EGF), 1 µg/mL Hydrocortisone, 10 mM Glutamine, FBS to a final concentration of 10%, 10 µg/ml gentamycin and 0.25 µg/ml amphotericin B

This thesis focuses on experiments (biological replicate n=3, technical replicate n=3) in BEWO, HTR8/SVneo, HMEC-1 and HUVEC models.

Briefly, cells were recovered from cryopreservation and thawed rapidly within 2 minutes in 37°C water bath by gentle agitation. Under strict aseptic precautions, cells were transferred to centrifuge tube containing complete culture media and spinned at 125xg for 5-7 minutes. The cell pellet obtained were resuspended with recommended complete media and dispensed into T75 culture flask. The culture was incubated in a humidified incubator at 37°C in a 5% CO2 in air atmosphere. The media was renewed after 24 hours of establishing a culture from cryopreserved cells. For subsequent subcultures, the media was changed every 48 hours until the culture is approximately 70-80% confluent. Once confluent, the culture media was discarded and the cell layer was rinsed with 1X PBS to remove all traces of serum, which contains trypsin inhibitor. Cells were incubated with 0.25% (w/v) Trypsin-0.53 mM EDTA solution at 37°C for 3 minutes. Cells were observed under an inverted microscope so as to confirm the entire cell layer to be detached. After adding complete growth media, cells were centrifuged and cell pellet was resuspended with recommended complete media. Around 20µl cell suspension were removed from the vial and was diluted in 20 µl of 0.4% trypan blue solution. Using a haemocytometer, number of viable cells per ml was determined.

% viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100

The cell suspension was diluted to a concentration of 25,000 viable cells/ml using the supplemented media.

5.2.1.2 Cryopreservation of cells

For long term storage, after trypsinization, cell pellet was resuspended in complete culture media supplemented with cryoprotective agent (7-10% DMSO). Cells were frozen in Mr Frosty containing isopropanol at -80^{o}C, which were subsequently transferred to liquid nitrogen after 24 hours.

5.2.1.3 Stimulants

Cells (BEWO/HUVEC) were treated with chemicals listed in table below for various time points depending on experiment protocol, which are explained in detail later in this report.

Table 8:

Concentration used for various stimulants during cell culture

Chemical reagents	**Vehicle**	**Stock Concentration**	**Desired Concentration**	**Storage**
Carbenoxolone	Water	100mM	100µM	Aliquots at -20°C
CRH	Water	1mM	100nM	Aliquots at -20°C
Forskolin	DMSO	100mM	100µM	Aliquots at -20°C
UCN2	5% Acetic acid	1mM	100nM	Aliquots at -20°C
VEGF	PBS	50µg/ml	20ng/ml	Aliquots at -20°C

5.2.2 **Western Blot**

5.2.2.1 Protocol

5.2.2.1.1 Soluble proteins preparation from cultured cells

All the steps involving sample preparation was carried on ice. Supernatants were collected for MSD assay and stored at -80^{o}C. Stimulation of cells was terminated by addition of ice cold PBS and cells were lysed with 100µl lysis buffer supplemented with protease and phosphatase inhibitors. Cells were immediately scraped off the plate and transferred into eppendorf and sonicated for 30 seconds for complete release of cellular proteins. After centrifugation at 13000 rpm for 15 minutes at 4^{o}C, protein concentration was determined using bicinchoninic acid (BCA) protein assay kit. Samples were prepared by mixing cell lysates with 5X loading dye and denatured at 95^{o}C for 10 minutes on heat block and then cooled immediately on ice. Samples were stored at -20°C until required.

5.2.2.1.2 Determination of protein concentration

The protein concentrations of lysates were determined using BCA Protein Assay Kit according to the manufacturer's manual. Protein lysates were diluted in 1 x RIPA and 1 in 5 dilutions was used for the assay. Protein concentration was determined by comparison of target samples to Bovine Serum Albumin (BSA) standard diluted in 1X RIPA buffer. 1X RIPA buffer was used as blank and was subtracted from each standard and sample. Tecan microplate reader (Magellan V5.03), Tecan, Switzerland, was used to determine the unknown protein concentration in the samples. The lysates were measured at 570nm absorbance.

5.2.2.1.3 SDS PAGE Electrophoresis

SDS PAGE mini gel glass plates were assembled according to manufacturer’s instruction. 10% resolving gel and 4% stacking gel were prepared.

Table 9:

Composition of 10% resolving gel and 4% stacking gel

10% Resolving gel	**2 gels**
Double Distilled Water	7.8ml
30% Acrylamide	6.6ml
Resolving buffer	5.2ml
10% APS	150µl
TEMED	15 µl

4% Stacking gel	**2 gels**
Double Distilled Water	6.1ml
30% Acrylamide	1.3ml
Stacking buffer	2.5ml
10% APS	50µl
TEMED	10 µl

10% resolving gel was poured between two glass plates, which were further topped up by a short 4% stacking gel. Once polymerized, gels were placed in running apparatus. Cell lysates were subjected to 10% SDS – PAGE for separation of protein mixtures by size. Biotinylated protein ladder was loaded to determine molecular weights. 25µg of cellular lysate was loaded per lane. Ishikawa cells were used as a known source of target protein. The positive control was used to confirm the activity of antibodies as well as identity of target protein since it will produce a reference band on the blot showing expected migration of target protein. The electrophoresis was run at 70V for initial 30 minutes, followed by 140V for 1 hour until the dye front had migrated to the bottom of the gel.

5.2.2.1.4 Transfer of separated proteins to a blotting membrane

Electrophoretic transfer was accomplished under wet conditions where the gel and nitrocellulose membrane were assembled into sandwich along with filter paper and fibre pad. The membrane was sandwiched between gel and positive electrode so that negatively charged proteins migrate from gel onto membrane. The cassette was immersed in a buffer tank containing 1x transfer buffer containing 20% methanol and transfer was run for 1 hour. After blotting, the apparatus was disassembled and the membrane was stained using reversible stain Ponceau S to confirm transfer. The membrane was thoroughly washed with 1x TBST (0.1% tween 20) until clear liquid is seen.

5.2.2.1.5 Immunodetection

After Ponceau S staining, the membrane was cut so as to separate into two blots – one for target protein and one for β-Actin, an internal control. β-Actin was used so as control for inconsistencies that arises during sample preparation, loading and transfer steps. The membrane was blocked for 1 hour at room temperature with LICOR blocking buffer in 1x TBS (1: 1). The membrane was further incubated with primary antibody diluted in 5% BSA in TBST overnight at 4^{o}C. Different dilutions (1 in 100, 1 in 250, 1 in 500, 1 in 1000, 1 in 2000, and 1 in 3000) were optimized for each antibody (data not shown).

Table 10:

List of primary antibodies used for western blot analysis

Primary antibody	**Catalogue number**	**Species**	**Dilution**	**Manufacturer**
AKT	2920	mouse	1 in 1000 in 5% BSA in TBST	Cell Signalling Technology, USA
Phospho AKT (ser 473)	4060	rabbit	1 in 1000 in 5% BSA in TBST	Cell Signalling Technology, USA
Phospho AKT (thr 308)	13038	rabbit	1 in 500 in 5% BSA in TBST	Cell Signalling Technology, USA
eNOS	5880	mouse	1 in 500 in 5% BSA in TBST	Cell Signalling Technology, USA
Phospho eNOS (ser 1177)	9570	rabbit	1 in 250 in 5% BSA in TBST	Cell Signalling Technology, USA
BCL 2	15071	mouse	1 in 250 in 5% BSA in TBST	Cell Signalling Technology, USA
β-Actin	8457	rabbit	1 in 3000 in 5% BSA in TBST	Cell Signalling Technology, USA
Phospho P38 MAPK (thr180/tyr182)	9216	mouse	1 in 250 in 5% BSA in TBST	Cell Signalling Technology, USA

p38 MAPK	8690	rabbit	1 in 2000 in 5% BSA in TBST	Cell Signalling Technology, USA
Phospho MTOR (ser 2448)	2971	rabbit	1 in 250 in 5% BSA in TBST	Cell Signalling Technology, USA
MTOR	2972	mouse	1 in 2000 in 5% BSA in TBST	Cell Signalling Technology, USA
Phospho CREB (ser133)	9198	rabbit	1 in 250 in 5% BSA in TBST	Cell Signalling Technology, USA
CREB	9197	mouse	1 in 2000 in 5% BSA in TBST	Cell Signalling Technology, USA
Phospho AMPK (Thr 172)	50081	rabbit	1 in 250 in 5% BSA in TBST	Cell Signalling Technology, USA
AMPK	2793	mouse	1 in 2000 in 5% BSA in TBST	Cell Signalling Technology, USA
HIF -1alpha	36169	rabbit	1 in 2000 in 5% BSA in TBST	Cell Signalling Technology, USA

Following day, the blots were washed three times for 10 minutes with 1X TBST at room temperature. The blots were incubated with secondary antibody for 1 hour at room temperature. Different dilutions (1 in 5000, 1 in 10,000, 1 in 15,000, and 1 in 20,000) were optimized for each antibody (data not shown).

Table 11:

List of secondary antibodies used for western blot analysis

Secondary Antibody	**catalogue number**	**Species**	**Dilution**	**Manufacturer**	**Detection method**
IRDye 680RD Goat anti-Mouse IgG (H+L)	926-68070	mouse	1 in 15,000 in LICOR buffer in 1x TBST	LI-COR Biosciences UK Ltd. ®, UK	Odyssey Imaging System
IRDye 800CW Goat anti-Rabbit IgG (H + L)	926-32211	rabbit	1 in 10,000 in LICOR buffer in 1x TBST	LI-COR Biosciences UK Ltd. ®, UK	Odyssey Imaging System
Peroxidase-Conjugated Goat Anti-Mouse Immunoglobulins	P044701-2	mouse	1 : 1000 in 5% milk in TBST	Dako UK Ltd, UK	ECL
Peroxidase-Conjugated Goat Anti-Rabbit Immunoglobulins	P044801-2	rabbit	1 : 1000 in 5% milk in TBST	Dako UK Ltd, UK	ECL

The membranes were washed two times for 10 minutes with 1X TBST and 1 time for 10 minutes with 1x TBS at room temperature.

5.2.2.1.6 Detection methods

5.2.2.1.6.1 Detection of proteins using LI-COR Odyssey Infrared Imaging System

Fluorescent signal intensity of phosphorylated AKT, eNOS, p38 MAPK, AMPK, CREB, MTOR and total AKT, eNOS, BCL-2, p38 MAPK, AMPK, HIF-1 alpha, CREB, expression was determined using Odyssey software version 3.0. For antibodies labelled with Alexa Fluor or IR 680, channel 700 was selected and for antibodies labelled with IR 800, channel 800 was used. Intensity 5 was used for imaging. Background subtraction was done from all the samples including loading controls during quantification. Image Studio™ Lite 5.x

software was used to measure average intensity of signal for each band. The phospho protein and total protein values were normalized to loading control (β-Actin, 42kDa) and then the data was expressed as phospho/total ratio. All images shown in this report are representative of biological replicate (n=3), technical replicate (n=3).

5.2.2.1.6.1.1 Stripping of membranes following LI-COR detection

Some of the blots were stripped and reprobed with antibody against total protein (data not shown). Membranes were washed two times for 15 minutes in ECL stripping buffer consisting of 25mM glycine buffer, pH 2 and 2% SDS at room temperature. Membranes were washed two times for 15 minutes with TBS, blocked again with LICOR Buffer in 1x TBS and reprobed with appropriate antibody.

5.2.2.1.6.2 Detection of proteins using enhanced chemiluminescense (ECL)

Phosphorylated and total AKT, eNOS, BCL-2 protein levels were initially detected using ECL method, as a part of optimization (data not shown). Later, LICOR imaging system was used to image membranes. Briefly, the membranes were blocked 5% milk in 1x TBS for 1 hour at room temperature, probed with primary antibody overnight at $4^{o}C$, washed 3x10 minutes with 1x TBST, probed with horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature. After 3 x 10 minutes wash with 1x TBST, membranes were incubated with ECL reagents as per manufacturer's instructions. The blots were detected using G: BOX Chemi XX6, Syngene, UK. Band densities were quantified using GeneSys v1.4.1.0 software. The membranes were stripped and reprobed with appropriate antibody.

5.2.2.1.6.2.1 Stripping of membranes following ECL detection

Membranes were incubated at 55°C in stripping buffer consisting of 62.5mM Tris HCL pH 6.8, 100mM β mercaptoethanol and 2% SDS for 30 minutes with occasional stirring. Membranes were further washed 3x 15 minutes with 1xTBST. Further steps were carried from blocking as described above.

5.2.2.2 Experimental protocol for western blot analysis of protein expression in BEWO

BEWO cells were plated in 6 well plates (50,000 cells / 2 ml/ well) in triplicates in complete growth media until 70% confluency. Heat inactivated FBS was used for all experiments related to forskolin to prevent the degradation of cyclic adenosine 3', 5'-monophosphate (cAMP). Cells were further serum starved overnight in growth media containing 0.2% FBS. Media was collected before (baseline) and after (SSM baseline) serum synchronization and stored at -80°C. Cells were stimulated with CRH (100µM) and forskolin (F; 100µM) and combination of both for 24 hours. Some cells were pretreated with CRH for 6hours, followed by presence or absence of CRH along with forskolin for 24hours. The procedure was exposed to low (3%O2) and high oxygen tension (21%O2). For some cells, co ells were exposed to low oxygen tension after pretreatment with CRH. Appropriate controls (H_20 and DMSO) were added at each time point. Cells were washed with 1X PBS before and after changing media throughout the experiment. All the supernatants were further collected and stored at -80^oC. The pretreatments were collected before adding stimuli and stored at -80^oC. The stimulation was terminated by addition of ice cold PBS. Protein levels of expression of different proteins were measured by using western blot analysis.

5.2.2.3 Experimental protocol for western blot analysis of activation and expression of proteins in BEWO

BEWO cells were plated in 6 well plates (50,000 cells / 2ml/ well) in triplicates in complete growth media until 70% confluency. Cells were further serum starved overnight in growth media containing 0.2% FBS. The next day, cells were stimulated with CRH (100nM) or UCN2 (100nM) for varying periods of time. Stimulation was terminated by addition of ice cold PBS. The western blot protocol was further followed. Protein levels of phosphorylation and expression were measured at each time point by using western blot analysis.

5.2.3 **MSD human angiogenesis panel assay**

VEGF, PLGF and sFLT-1 biomarker levels were determined in cell free supernatants using angiogenesis panel assay kit according to manufacturer's instructions www.mesoscale.com/~/media/files/product%20inserts/angiogenesis%20panel%201%20human%20insert.pdf. This assay is based on sandwich immunoassay principle. Briefly, MSD plate was pre coated with capture antibodies on independent and well defined spots. The nonspecific binding sites were blocked and antigen containing samples were added to the plate. Samples were brought to room temperature and centrifuged at 1000 rpm for 10 minutes before adding to plate. Detection antibodies conjugated with electrochemiluminescent labels were further added. Analytes in the samples bind to capture antibodies immobilized on working electrode surface, recruitment of detection antibodies by bound analytes complete the sandwich. The read buffer was added to create appropriate chemical environment for electrochemiluminescence and the plate is inserted into MSD SECTOR Imager 6000 (Meso Scale Discovery (MSD), Gaithersburg, MD, USA). The intensity of emitted light was measured to provide quantitative measure of proteins in sample. Different dilutions (1: 4, 1:16, 1: 20, 1:2) of samples were tested and optimized for the assay. 1 in 20 dilutions was chosen for BEWO samples and 1 in 4 dilutions was used for HUVEC samples. Samples were tested in duplicates. Data in each sample was normalized against total amount of cellular protein and was expressed as ng/ml/mg protein or pg/ml/mg protein.

Dynamic Range

The concentrations of calibration curve for each assay are optimized for a maximum dynamic range while maintaining enough calibration points near the bottom of the curve to ensure a proper fit for accurate quantification of samples that require high sensitivity.

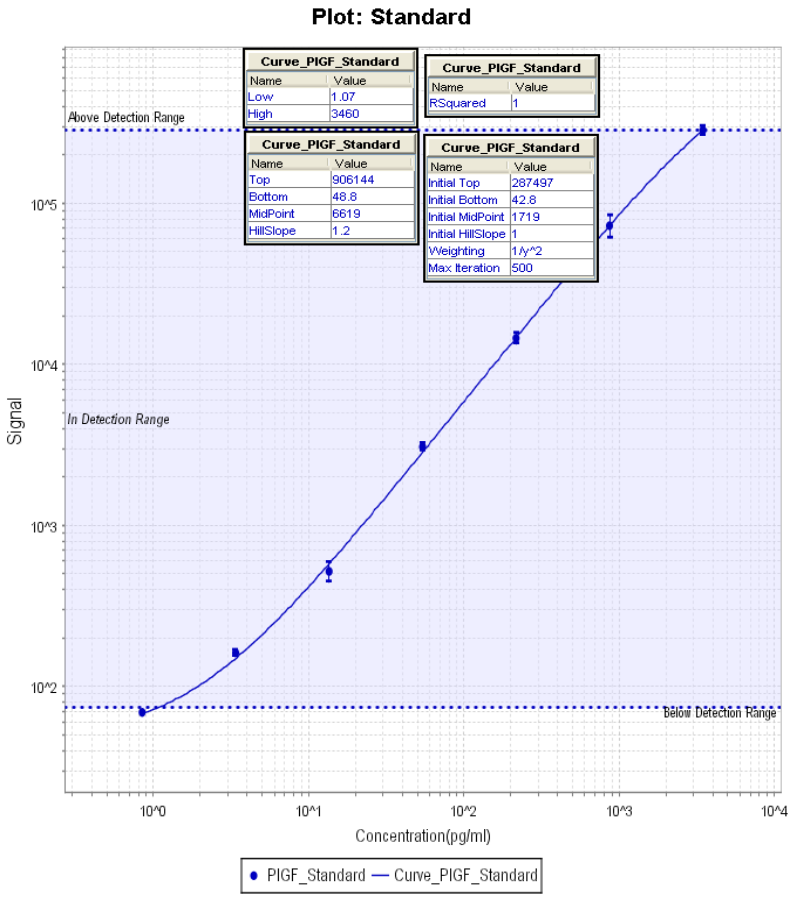


Figure 7

Standard curve of PLGF ELISA assay.

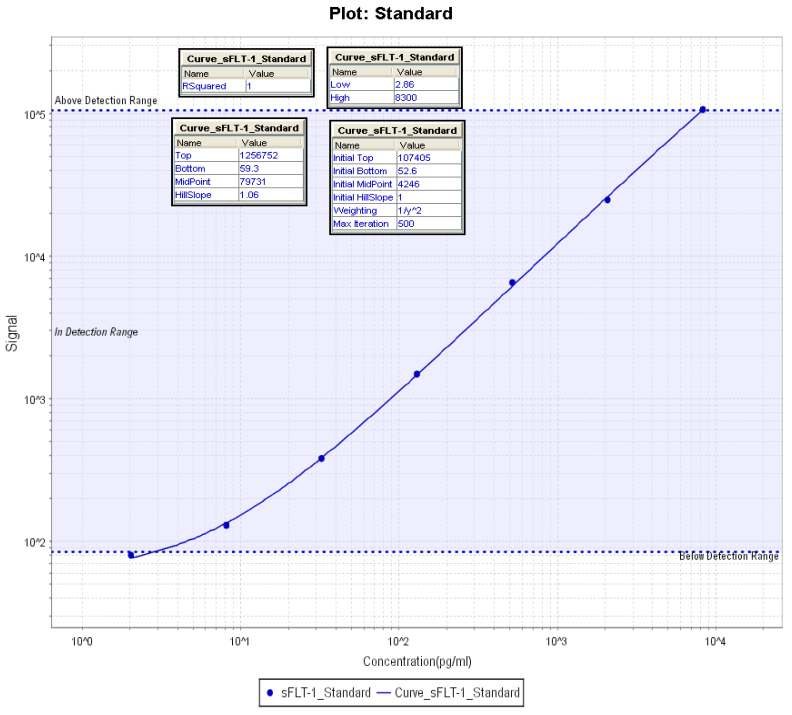


Figure 8

Standard curve of sFLT-1 ELISA assay.

Sensitivity

The lower limit of detection (LLOD) is a calculated concentration corresponding to the average signal 2.5 standard deviations above the background (zero calibrator).

The LLOD is a calculated concentration corresponding to the signal 2.5 standard deviations above the background (zero calibrator).

The ULOQ is the highest concentration at which the CV of the calculated concentration is <20% and the recovery of each analyte is within 80% to 120% of the known value.

The LLOQ is the lowest concentration at which the CV of the calculated concentration is <20% and the recovery of each analyte is within 80% to 120% of the known value.

The quantitative range of the assay lies between the LLOQ and ULOQ.

Accuracy and Precision: For most assays, control measurements results are within 20% of the expected concentration for each run. Precision is stated as the coefficient of variation (CV). Intra-run CVs fall below 7%, and inter-run CVs are typically below 15%.

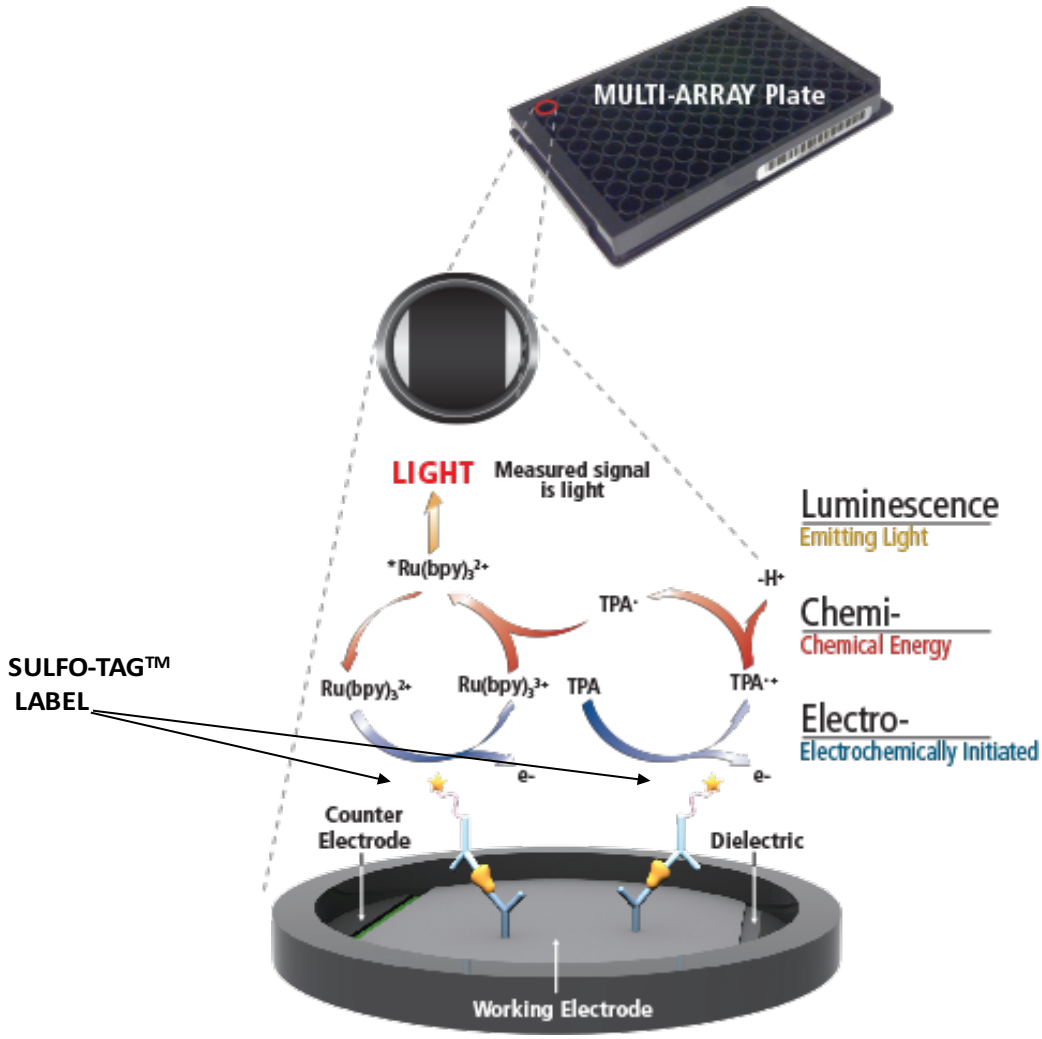


Figure 9

MSD assay principle (https://www.mesoscale.com/en/technical_resources/our_technology/ecl)

5.2.3.1 Experimental protocol for MSD assay

Cells (HUVEC/BEWO) were plated in 6 well plates (50,000 cells / 2ml/ well) in triplicates in complete growth media until 70% confluency. Cells were further serum starved overnight in growth media containing 0.2% FBS. Media was collected before (baseline) and after (SSM baseline) serum synchronization and stored at -80°C. The baselines were assessed for BEWO but not HUVECs. VEGF (20ng/ml) was used as a positive control to produce the expected effect with the assay or experimental set up. Cells were stimulated with CRH (100nM), UCN2 (100nM), combination of CRH or UCN2 with VEGF, at designated time points. Appropriate controls (H_20, 5% acetic acid, PBS) were added for

each time point. Cell free supernatants were collected and stored at -80^{o}C. Some wells in HUVEC culture were pretreated with CRH or UCN2 for 6 hours, which was further followed by various protocols for all the indicated time points (a) CRH or UCN2 in presence of VEGF [{CRH (CRH+VEGF)} or {UCN2 (UCN2+VEGF)}]; (b) VEGF only [CRH (VEGF) or UCN2 (VEGF)]. Cells were washed with 1X PBS before and after changing media throughout the experiment. The pretreatments were collected before adding stimuli and stored at -80^{o}C. The stimulation was terminated by ice cold PBS. Cell lysates were collected and protein concentration was determined. The frozen supernatants were thawed, brought to room temperature and sFLT-1, VEGF and PLGF release was determined by MSD assay.

5.2.4 **Electrochemiluminescence immunoassay (ECLIA)**

ECL (ElectroChemiLuminescence) is Roche's technology for immunoassay detection www.cobas.com/content/dam/cobas_com/pdf/product/Elecsys%20Calcitonin/Elecsys %20Calcitinin%20Fact%20Sheet.pdf. The development of ECL immunoassays is based on the use of a ruthenium-complex and tripropylamine (TPA). The chemiluminescence reaction for the detection of the reaction complex is initiated by applying a voltage to the sample solution resulting in a precisely controlled reaction.

5.2.4.1 Experimental protocol for Electrochemiluminescence immunoassay (ECLIA)

-BEWO cells were treated with appropriate stimulant (CRH 100µM and forskolin (F) 100µM and combination of both for 24 hours. Some cells were pretreated with CRH for 6 hours followed by forskolin with or without CRH for 24 hours. The experiment was exposed to low (3% O2) and high oxygen tension (21% O2). Some cells were exposed to low oxygen tension (3% O2) after the pretreatment with CRH. Heat inactivated FBS was used for all experiments related to forskolin to prevent the degradation of cAMP. The supernatant as well as pretreatments were collected after 24 hours of incubation and centrifuged at 13,000 rpm for 5 min at 4^{o}C. Supernatants without cell debris were transferred to fresh eppendorfs and stored at -80^{o}C until analysis. Before analysis,

supernatants were thawed and brought to room temperature. Endocrine differentiation was assessed by measuring BEWO cells' capacity to secrete β-HCG, progesterone and estradiol. 1 in 10 dilutions was used for progesterone analysis. β-HCG, progesterone and estradiol concentration was analyzed by fully automated modular analytics E170 testing system from Roche Diagnostics (Mannheim, Germany) by department of Biochemistry at University Hospital Coventry and Warwickshire NHS trust.

5.2.5 Proteome Profiler Human Angiogenesis Array

A membrane-based multiplex antibody array for the parallel analysis of the relative levels of selected proteins related to angiogenesis. It is validated for analyte detection in cell culture supernates, cell lysates, tissue lysates, serum, plasma and saliva. The essential benefits include its capacity to detect 55 human angiogenesis-related proteins simultaneously and is compatible with detection methods such as LI-COR and chemiluminescence. The advantages of the array are its ability to detect multiple proteins in a single sample and thus be cost-effective and also save time and sample.

5.2.5.1 Principle of the Assay

The Proteome Profiler Human Angiogenesis Array Kit is a membrane-based sandwich immunoassay. A cocktail of biotinylated detection antibodies is added to the sample and then array membrane which is spotted in duplicate with capture antibodies to specific target proteins are incubated in them. Captured proteins are visualized using chemiluminescent detection reagents. The signal produced is proportional to the amount of bound analytes including extracellular matrix components, membrane-bound receptors, soluble growth and differentiation factors, proteases, and intracellular signaling molecules.

5.2.5.2 Experimental protocol for Human Angiogenesis Array

All reagents were brought to room temperature before use. Samples were placed on ice. The procedure was followed as per the manual instructions. Human Angiogenesis Array detects relative expression levels of individual analytes. Appropriate control samples were included. Sample amount were empirically adjusted to attain optimal sensitivity

with minimal background. 250 µg for cell lysates were used. Control and CRH treated samples under high (21% O2) and low (3%O2) oxygen tension were used. Cell lysates were rinsed with PBS, making sure to remove any remaining PBS before adding lysis buffer. Cells at 1 x 10cells/mL were solubilized in lysis buffer and were pipetted up and down to resuspend and rock the lysates gently at 2-8 °C for 30 minutes. Cells were microcentrifuged at 14,000 x g for 5 minutes, and supernate was transferred into a clean test tube. Quantitation of sample protein concentrations using a total protein assay was done. Assay was performed. 2.0 mL of Array Buffer 7 was pipetted into each well of the 4-Well Multi-dish to be used. Array Buffer 7 was served as a block buffer. Using at-tip tweezers, each membrane to be used was removed from between the protective sheets and place in a well of the 4-Well Multi-dish and incubated for one hour on a rocking platform shaker. While the membranes are blocking, samples were prepared by adding up to 1.0 mL of each sample to 0.5 mL of Array Buffer 4 in separate tubes. 15 µL of reconstituted Detection Antibody Cocktail were added to each prepared sample, mixed and incubated at room temperature for one hour. Array Buffer 7 from the wells of the 4-Well Multi-dish was aspirated and sample/antibody mixtures was prepared as described above and incubated overnight at 2-8 °C on a rocking platform shaker. Each membrane was carefully removed and placed into individual plastic containers with 20 mL of 1X Wash Buffer. The 4-Well Multi-dish with deionized or distilled water was rinsed and dry thoroughly. Each membrane was washed with 1X Wash Buffer for 10 minutes on a rocking platform shaker. This was repeated two times for a total of three washes. IRDye-streptavidin was diluted in Array Buffer 5 using the dilution factor on the vial label. 2.0 mL of diluted IRDye-streptavidin into each well of the 4-Well Multi-dish was pipetted. Each membrane was incubated for 30 minutes at room temperature on a rocking platform shaker. Membrane was washed and placed on the bottom sheet of a plastic sheet protector.

5.2.5.3 Data analysis

Fluorescent signal intensity of multiple protein expression was determined using Odyssey software version 3.0. Channel 800 was used. Intensity 5 was used for imaging. The average signal (pixel density) of the pair of duplicate spots representing each angiogenesis-related protein was determined. An averaged background signal was

subtracted from each spot. A signal from a clear area of the array or negative control spots as a background value was used. Corresponding signals on different arrays to determine the relative change in angiogenesis-related proteins between samples were compared. Histogram profiles for select analytes were generated by quantifying the mean spot pixel densities from the array membrane using image software. String software (Szklarczyk et al. Nucleic Acids Res. 2015 43(Database issue):D447-52) was further used to assess protein- protein interaction networks.

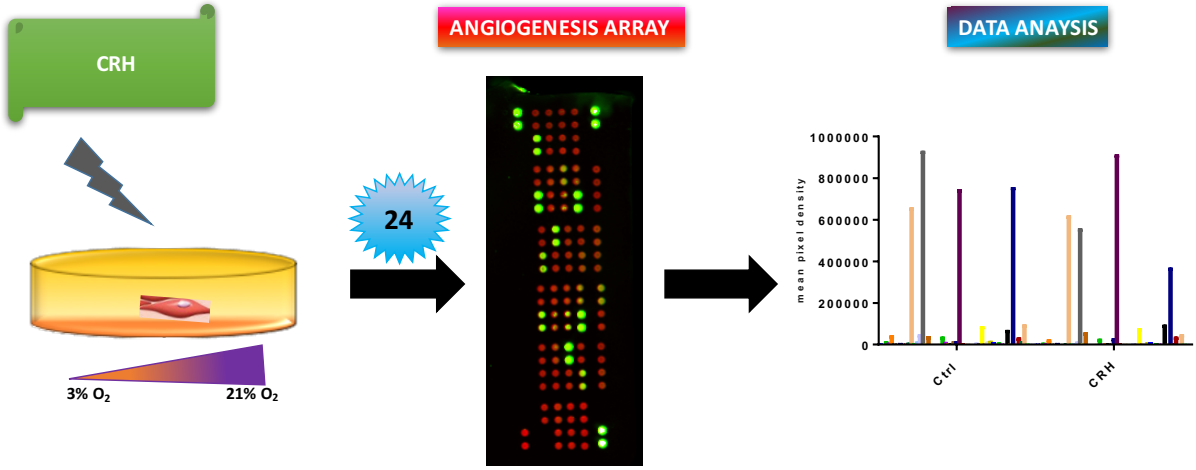


Figure 10

Angiogenesis array protocol

5.2.6 QRT PCR

5.2.6.1 RNA isolation

5.2.6.1.1 Protocol

Sigma's GenElute Mammalian Total RNA Miniprep Kit provides a simple and convenient way to isolate total RNA from mammalian cells and tissues. The kit combines the advantages of a silica-based system with a microspin format and eliminates the need for cesium chloride gradients, alcohol precipitation, and hazardous organic compounds such as phenol and chloroform. Cells or tissues are lysed and homogenized in a buffer containing guanidine thiocyanate to ensure thorough denaturation of macromolecules

and inactivation of RNAses. Conditions during column binding and the rst wash remove RNAses. Addition of ethanol causes RNA to bind when the lysate is spun through a silica membrane in a microcentrifuge tube. After washing to remove contaminants, RNA is eluted in 50–100 µL of Elution Solution.

The RNA isolation was carried as per the protocol associated with the kit. All steps are carried out at room temperature except where otherwise noted. All spins were carried at 14000 x g. Briefly, 250 µL of the Lysis Solution/2-ME Mixture for up to 10 cm^2 of surface area was added and left for 2 minutes to lyse the cells and RNA is released. So as to enable RNA to bind to column, the lysate is transferred to filtration column and spinned for 2 minutes. Filtration column is discarded. Equal volume of 70% ethanol is added to filtrate. the mixture is transferred to to binding column and spinned for 15 seconds. The flow-through is discarded & repeat if necessary. So as to remove contaminants, 250 µL Wash Solution 1 to column was added and spinned for 15 seconds. Even minor DNA contamination can give false positive detection for sensitive applications such as QRT PCR. Significantly lower levels of DNA contamination can be achieved by digesting with DNase I using the On-Column DNase I Digestion Set (DNASE10 and DNASE70). 10 µL of DNase I (D2816) with 70 µL of DNase Digest Buffer (D1566) for each preparation was mixed. 80 µL of the DNase I/Digest Buffer mixture directly onto the lter in the Binding Column was added and incubated at RT for 15 minutes. 250 µL Wash Solution 1 to column was added and spinned for 15 seconds. The column is transferred to new collection tube. 500 µL Wash Solution 2 to column was added. The tube was spinned for 15 seconds and Wash Solution was discarded. Second 500 µL Wash Solution 2 to column was added and spinned for 2 minutes to remove ethanol. So as to elute purified RNA, column was transferred to new collection tube. 50 µL elution solution to column was added and spinned for 1 minute.

5.2.6.2 cDNA synthesis

5.2.6.2.1 Protocol of cDNA synthesis

High capacity RNA-to-cDNA kit Applied Biosystems (Fisher Scientific; Part Number: 4387406) was used. Upto 2 µg of total RNA per 20-µL reaction. RT reaction mix (20-µL

per reaction) using the kit components before preparing the reaction plate/tubes was prepared. The kit components to thaw on ice.

Table 12:

List of CDNA synthesis components

Component	Volume/ Reaction (μL)	
	Sample	**Control**
2x RT Buffer	10	10
20x RT Enzyme Mix	1	-
Nuclease-free water	*Variable	*Variable
RNA sample	*25ng-2μg (<9 μL)	*25ng-2μg (<9 μL)
Total per Reaction	**20μL**	**20μL**

11 µL of Master Mix was aliquoted into each reaction tube. Respective amount of RNA sample into reaction tube was added and made upto the volume to 20 µL by adding enough nuclease- free water and briefly centrifuge the tubes to spin down the contents and to eliminate air bubbles. The tube on ice was placed until ready to load into thermal cycler. The thermal cycler conditions were programed according to table below and cDNA tube was stored in -15 to -25 freezer.

Table 13:

Thermal cycler conditions for cDNA synthesis

	Step 1	**Step 2**	**Step 3**
Temperature (Celsius)	37	95	4
Time	60 min	5 min	-

5.2.6.3 Real Time Quantitative PCR (qPCR)

Protocol

cDNA was diluted with molecular grade nuclease free H2O (check if it needs DEPC-treated or not) to **1:5** (most commonly used) or **1:3** in the labelled PCR tube. The tubes were spinned briefly to mix and bring down the solution. Total volume in single well should be = 20µl. (multiple by 2 if to make duplicates).

20µl = 10 µl SYB Green
1 µl Forward Primer
1 µl Reverse Primer
1 µl diluted cDNA
7 µl nuclease -free H2O.

All these were prepared in 1.5ml tube for all samples including duplicates, one for each gene. Therefore, e.g. for 10 well (duplicates included): 11x19 µl. 19 µl of master mix prepared was loaded above in each respective well and 11 µl cDNA was added. The plate was covered with sticky plastic film and spinned down at 300g for 3 min. The plate was run on the FAST 7500 qPCR machine.

5.2.6.3.1.1 Real-time PCR primer design

Primers for mRNA were synthesized by Sigma Aldrich and 100 uM stocks were prepared by dissolving the dried nucleic acids in nucleic acid nuclease-free water and stored at -20 °C. Design was performed using Ensemble Genome Browser Primers tool (http://www.ensembl.org/index.html) or Primer 3 (http://simgene.com/Primer3). Primer characteristics were evaluated through USCS Genome In-silico PCR **(http://genome.uscs.edu/cgi-bin/hgPcr).** The amplicon length should be approximately 50–150 bp, since longer products do not amplify as efficiently. In general, primers should be 18–24 nucleotides in length. This provides for practical annealing temperatures. Primers should be designed according to standard PCR guidelines. They should be specific for the target sequence and be free of internal secondary structure. Primers should avoid stretches of homopolymer sequences (e.g., poly(dG)) or repeating motifs, as these can hybridize inappropriately. Primer pairs should have compatible melting temperatures (within 1°C) and contain approximately 50% GC content. Primers with high GC content can form stable imperfect hybrids. Conversely, high AT content depresses the Tm of

perfectly matched hybrids. If possible, the 3' end of the primer should be GC rich to enhance annealing of the end that will be extended. Analyze primer pair sequences to avoid complementarity and hybridization between primers (primer-dimers).

For qRT-PCR, primers were designed that anneal to exons on both sides of an intron (or span an exon/exon boundary of the mRNA) to allow differentiation between cDNA amplification and potential genomic DNA contamination by melt curve analysis. BLAST® search was performed against public databases to be sure that the primers only recognize the target of interest and thus confirm specificity of primers. A final concentration of 200 nM for each primer was assessed to be effective for most reactions.

Table 14:

List of Primers

Gene (Human)	**Forward Sequence**	**Backward Sequence**
Syncytin 1	CCCCATCGTATAGGAGTCTT	CCCCATCAGACATACCAGTT
Syncytin 2	GCCTGCAAATAGTCTTCTTT	ATAGGGGCTATTCCCATTAG
CRHR 1 a/b	GGCAGCTAGTGGTTCGGCC	5TCGCAGGCACCGGATGCTC
CRHR 2a	GAGCTGCTCTTGGACGGC	GACAAGGGCGATGCGGTA
VEGF	TCGGGCCTCCGAAACCATGA	CCTGGTGAGAG ATCTGGTTC
PLGF	GCCTGGATGAGAAACAGCTC	GAGAATCTGGCTTGGCAGTC
sFLT-1	CTAATTGTCAATGTGAAACC CCAG	TATGTTTCTTCCCACAGTCCCAAC
ICAM-1	GGCCGGCCAGCTTATACAC	TAGACACTTGAGCTCGGGCA
VCAM-1	TCAGATTGGAGACTCAGTCATGT	ACTCCTCACCTTCCCGCTC
B actin	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

5.2.6.3.2 Data analysis Pt 1

Fold-change calculations. ΔΔCt method was employed.

a) ΔΔCt assumes that each reaction cycle gives a doubling of gene product, i.e. the PCR reaction is perfectly efficient for both GoI and reference gene. It calculates the difference in Ct value between reference and GoI (ΔCt) and compares this between a sample and a calibrator/control (ΔΔCt) or alternatively to an average ΔCt value for the complete sample set. It uses the equation:

   $2^{(CtGoI-CtRef)\ cont\ -\ (CtGoI-CtRef)\ test}$ where 'cont' is control/calibrator sample (or average value) and 'test' is individual sample being analyzed.

- The ΔΔCt equation is also rearranged to allow separate efficiency values for GoI and ref genes.

  With the assumption of equal primer efficiency, double delta Ct analysis is used to test large amounts of DNA samples and a low number of genes. The average of the Ct values for the housekeeping gene and the gene being tested in the experimental and control conditions is assessed. The 4 values were Gene being Tested Experimental (TE), Housekeeping Gene Experimental (HE), Gene being Tested Control (TC), and Housekeeping Gene Control (HC). The differences between TC and HC (TC-HC) and TE and HE (TE-HE) are calculated so as to obtain ΔCt values for the experimental (ΔCTE) and control (ΔCTC) conditions, respectively. Then, the difference between ΔCTE and ΔCTC (ΔCTE-ΔCTC) is calculated to arrive at the Double Delta Ct Value (ΔΔCt). All the calculations are in logarithm base 2. Thus, each time when there is twice as much DNA, the Ct values will not halve but rather decrease by 1. So as to get the expression fold change, the value of 2^-ΔΔCt was calculated.

5.2.6.3.2.1 Melting curve (dissociation curve)

A melting curve plots the change in fluorescence seen when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates ("melts") into single-stranded DNA (ssDNA) as the temperature of the reaction is raised. Post-amplification melting-curve analysis is a straightforward and simple way to investigate real-time PCR reactions for primer-dimer artifacts and to ensure specificity of a reaction.

5.2.6.3.3 Data analysis Pt 2 – basic stats

In general, though, for detection efficiency purposes it is best to use parametric methods where possible, provided the data meets requirements of normality and homogeneity of variance or can be transformed to do so. Experimental analysis statistics were conducted on raw or normalized Ct value and not on fold-change values. Gene fold-change data is typically not normally distributed. However, log transformation is usually sufficient to give an approx. normal distribution, which incidentally brings it down to the original Ct scale.
Typical parametric analyses for column-style data will involve t-test or ANOVA. Welch's correction allows normally distributed data with heterogeneous variance to be analyzed using these methods. For QRT PCR, standard deviation of the 2^-ddCT values of all the replicates were taken and divided the mean of the 2^-ddCT value of the control while presenting the data as fold change.

5.2.6.3.3.1 Interpreting data

Some considerations:

- When comparing tissues at different developmental stages or following different treatments, does a GoI fold-change reflect a 'true' change in cellular expression level or is it a secondary result of a change in cell population? (e.g. Inflammatory cell influx at labour).

- When choosing a reference gene under conditions where profound changes in global mRNA expression patterns occur (e.g. altering metabolism/proliferation), normalization strategies are not straightforward. For example, if global mRNA synthesis is elevated then a reference that appears to stay constant, having already normalized the sample for RNA concentration, will really be one whose levels are relatively increased within the cellular pool. Whether this matters depends what you want from the data.
- Similarly, changes in tissue mass due to e.g. increased fluid will result in lower effective protein concentrations invisible at the qPCR level. Overall, with most ligand-receptor or enzymatic reactions it is component concentration, not even total amount that governs functional effects.
- Is the protein of interest regulated at the level of steady-state mRNA expression?

5.2.7 **Statistical analysis**

In this thesis, all data are expressed as mean ± S.D (Standard deviation). Whether the data was distributed normally or not was assessed by Shapiro Wilk normality test. The effects of treatments, and their combinations at various time points were compared and analyzed by one way or two way ANOVA with tukeys multiple comparison method using graph pad software (San Diego, CA, USA). Data was analyzed with student's t test or Mann Whitney test where applicable. Differences were considered to be statistical significant at a value of $p < 0.05$.

6 Results

6.1 BEWO

6.1.1 The effect of hypoxia on the expression of fusogenic genes

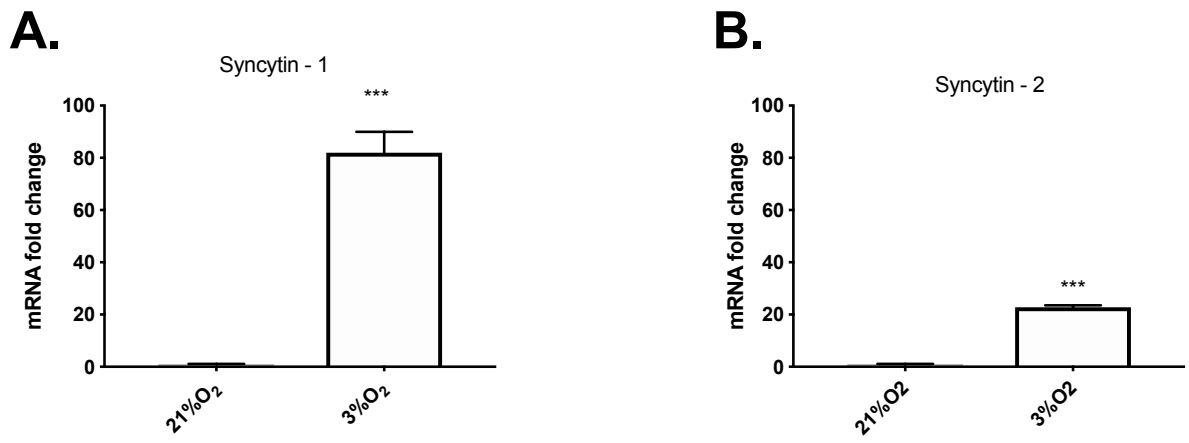


Figure 11

*Hypoxia increases syncytin 1 and 2 MRNA expression in BEWO cells.QRT PCR analysis showed that the expression of (A) syncytin 1 and (B) syncytin 2 mRNA was significantly upregulated for 24h at 3% O2. *** p=.0002; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.*

6.1.2 The expression of fusogenic genes in syncytialised bewo cells

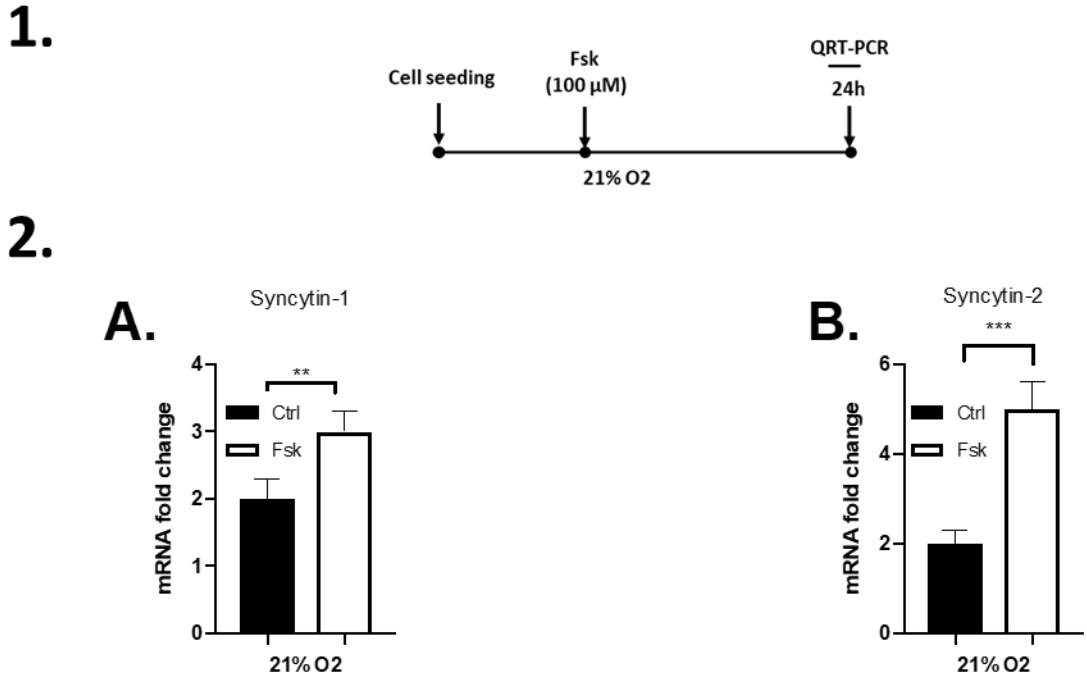


Figure 12

*CAMP/PKA signalling pathway increases syncytin 1 and 2 MRNA expression in BEWO cells. 1. Experiment protocol; where after 80% confluency, BEWO cells are treated with CAMP agonist for 24h. 2. QRT PCR analysis showed that the expression of (A) syncytin 1 and (B) syncytin 2 mRNA was significantly upregulated for 24h at 21% O2. ** .002 , *** p=.0002; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.*

6.1.3 The effect of hypoxia on the expression of fusogenic genes in syncytialised bewo cells

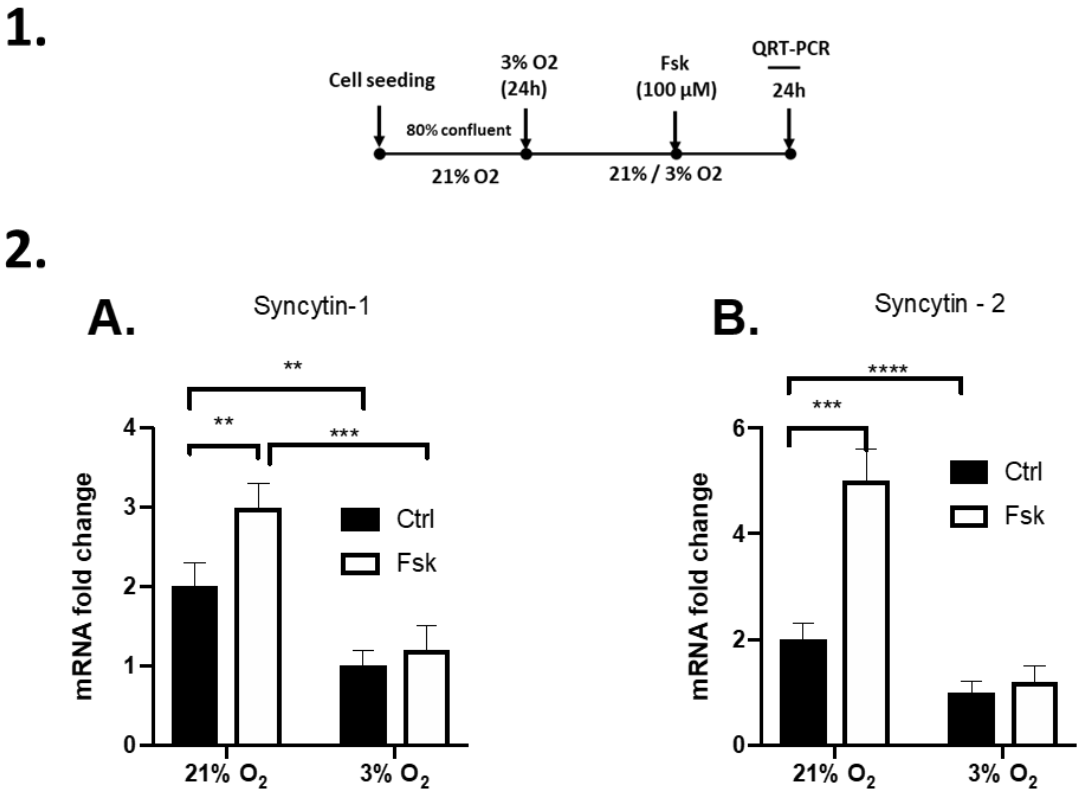


Figure 13

*Low oxygen tension impairs syncytin 1 and 2 gene expression induced by CAMP signalling pathway in BEWO cells. 1. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were treated with 100um forskolin for 24h. 2. QRT PCR analysis showed that low oxygen tension abolished upregulation of (A) syncytin 1 and (B) syncytin 2 induced by CAMP agonist. p= ** .002 *** .0002 ****< .0001; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using using two – way ANOVA with Tukeys multiple comparison test.*

6.1.4 Phospho CREB subcellular distribution induced by forskolin in BEWO cells : visualization by confocal microscopy

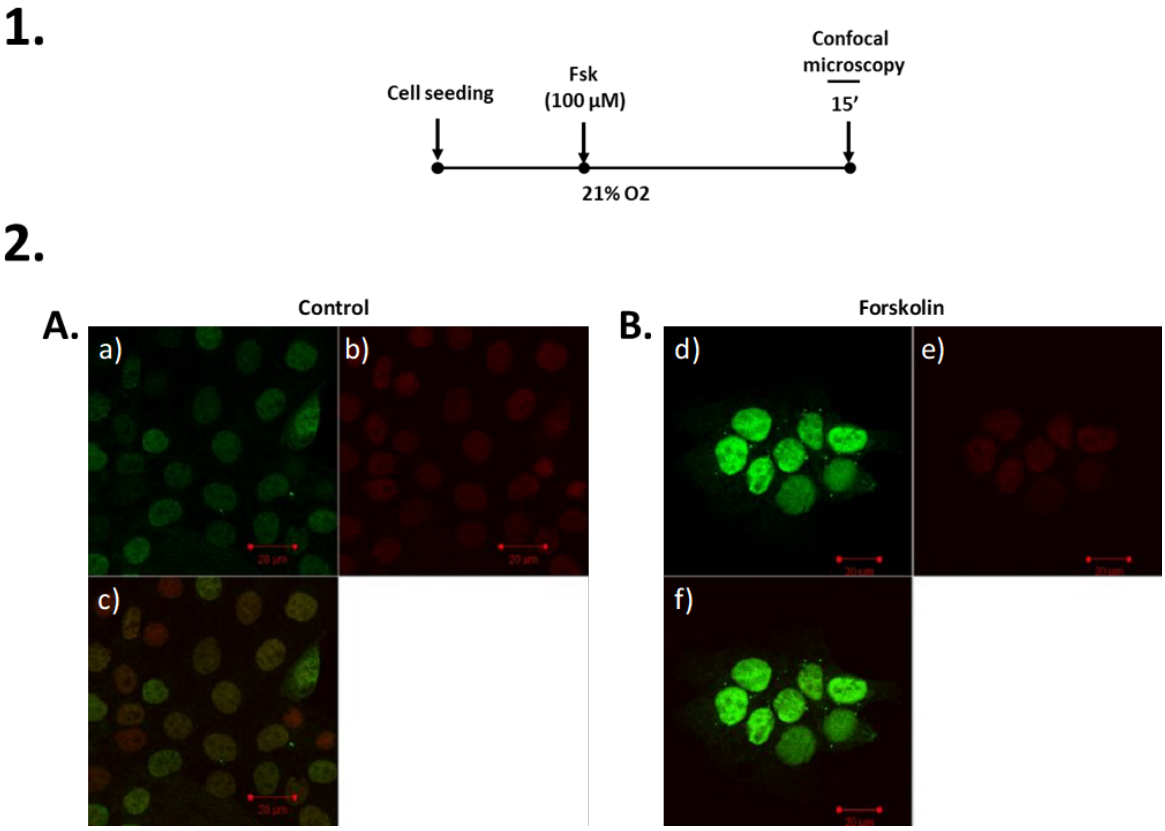


Figure 14

Forskolin induces BEWO cell fusion. Representative confocal microscopic images of sorted unfused BEWO cells and fused BEWO cells. 1. Experiment protocol; where after 80% confluency, cells were incubated with 100 µM forskolin for 15' and phospho –CREB Ser133 subcellular distribution in BEWO cells were detected using confocal microscopy. Phospho CREB (green) and CREB (red) distribution was monitored over 15' by indirect immunofluorescence using specific primary antibodies and FITC mouse 488 and Rabbit 563 secondary antibodies. Cell nuclei were stained with the DNA specific dye DAPI (blue) (data not shown). 2.(B) Confocal microscopy confirmed BEWO cells were mainly composed of fused cells characterised by a multinucleated syncytium. (A) In contrast, in absence of forskolin, the cell population contained mono-nucleated syncytium that displayed no change in cell size. Scale bar,20 µM; n=3.

6.1.5 The effect of hypoxia on the release of B-HCG, Progesterone and Cortisol in BEWO cells

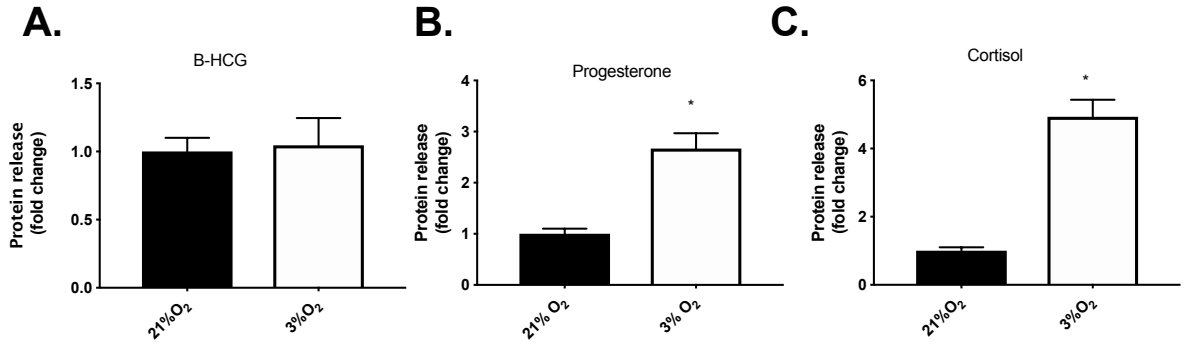


Figure 15

*Hypoxia increases progesterone and cortisol release, but not B-HCG release in BEWO cells. ECLIA analysis showed that the release of (B) progesterone and (C) cortisol but not (A) B-HCG was significantly increased at 3% O2 at 24h. *p= 0.033; n=3. Data expressed as fold change of protein release, relative to control 24h, after normalising against IU/mg protein. Data were analysed using a two-tailed unpaired t-test.*

6.1.6 The secretion of B-HCG, Progesterone, Estradiol and Cortisol in culture medium of BEWO cells in response to CAMP agonist

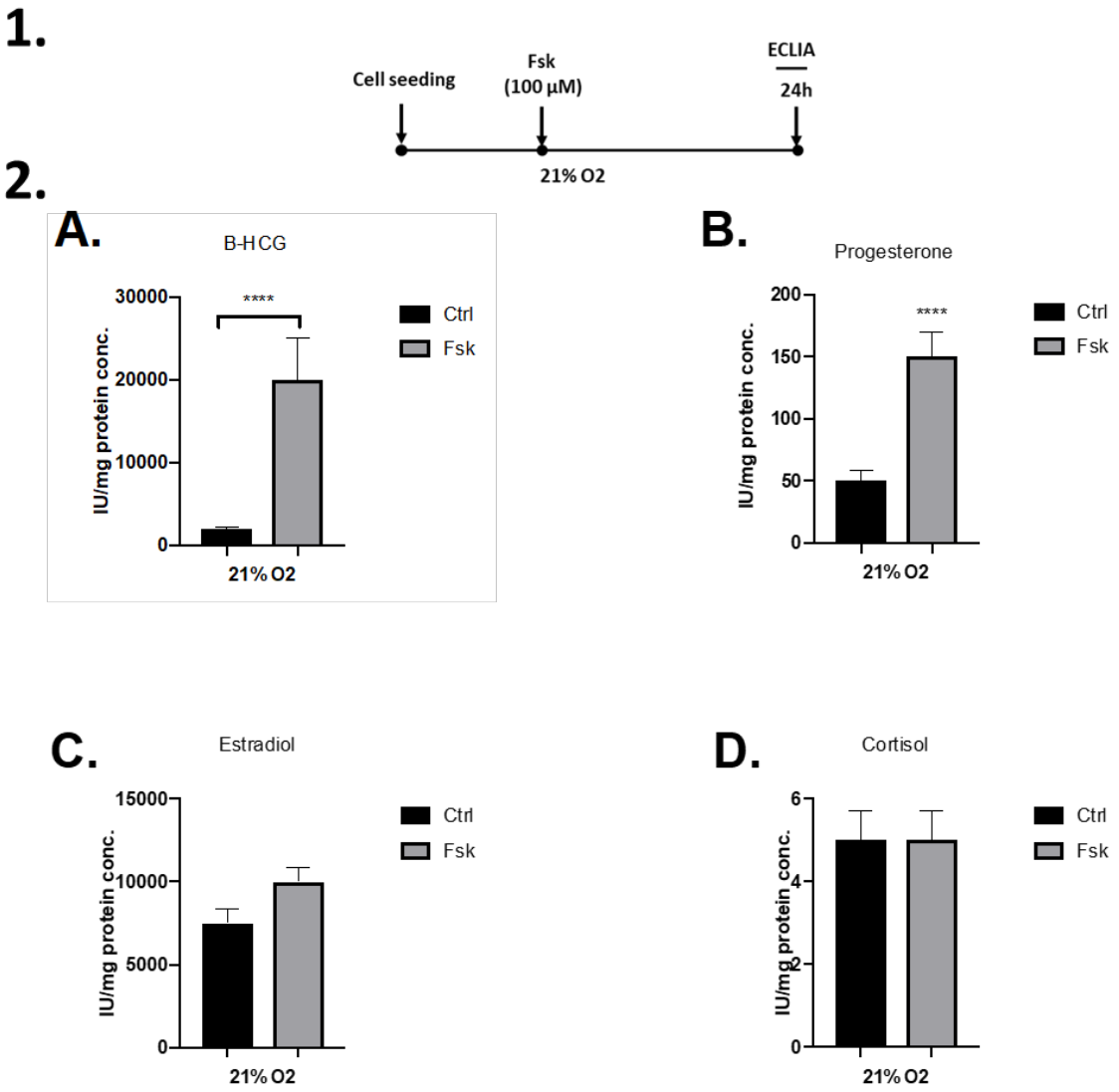


Figure 16

*CAMP/PKA signalling pathway increases protein release of B-HCG, progesterone but not estradiol and cortisol in BEWO cells. 1. Experiment protocol; where after 80% confluency, BEWO cells are treated with CAMP agonist for 24h. 2. MSD ECLIA assay showed that the release of (A) B-HCG and (B) progesterone, but not (C) estradiol and (D) cortisol was significantly increase for 24h at 21% O2. ****p< .0001; n=3. Data expressed as IU/mg protein concentration, relative to control 24h. Data were analysed using a two-tailed unpaired t-test.*

6.1.7 The effect of hypoxia on the endocrine capacity and biochemical differentiation in syncytialised BEWO cells

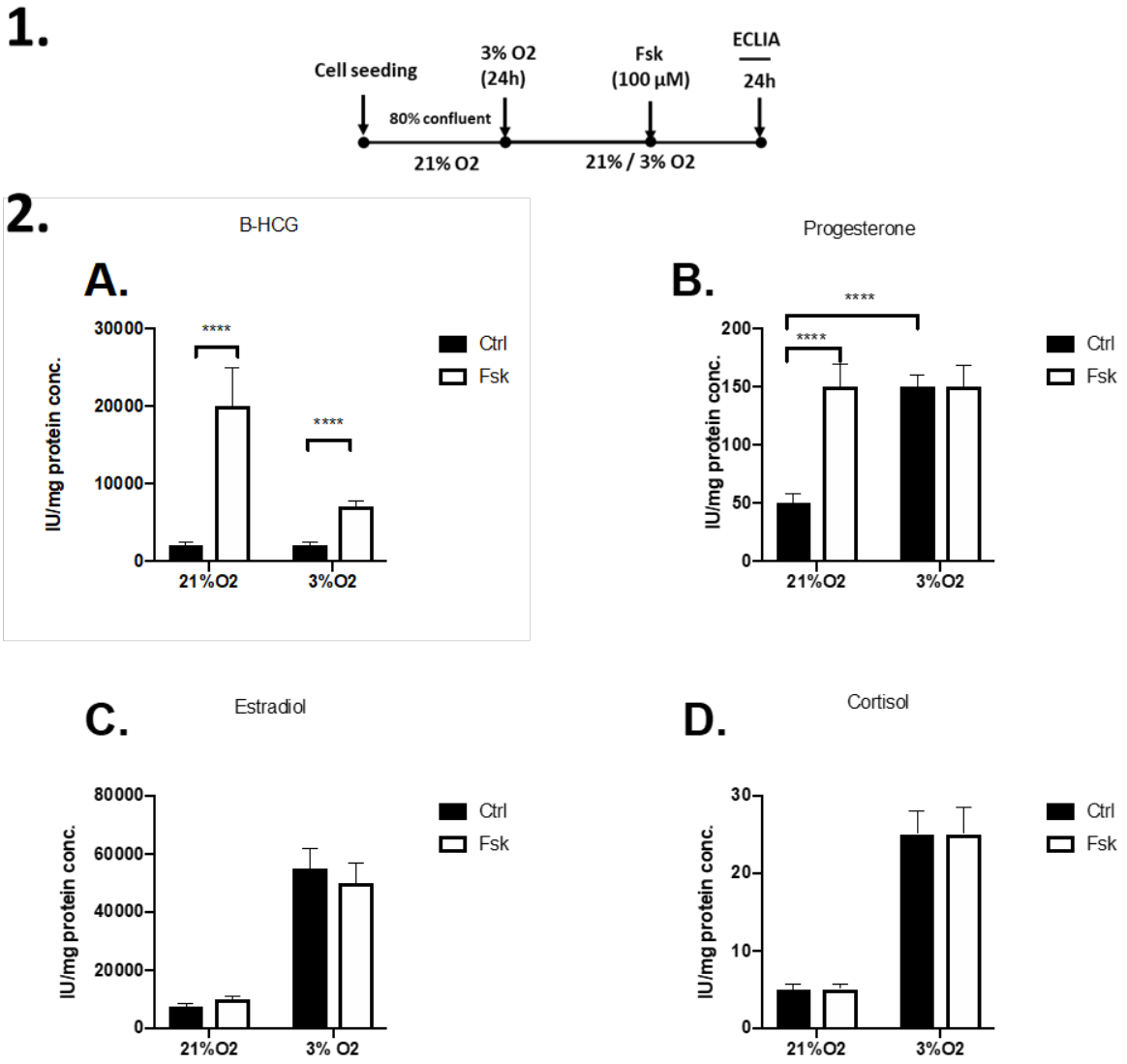


Figure 17

*Low oxygen tension impairs the enhanced release of B HCG and progesterone induced by cAMP signalling pathway in BEWO cells. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were treated with 100um forskolin for 24h. 2. ECLIA analysis showed that low oxygen tension impaired enhanced release of B HCG and abolished progesterone release induced by CAMP agonist. Estradiol and cortisol release remained unaffected by low oxygen tension. ****p< .0001; n=3. Data expressed as IU/mg protein concentration, relative to control 24h. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

To determine the effect of the oxygen tension on the cell fusion, BeWo cells were cultured in either 21% or 3% oxygen in the presence or absence of forskolin and then the mrna expression of syncytin 1 and 2 were measured. Forskolin, a small lipophilic molecule easily to be absorbed

and distributed, is a well-known adenylate cyclase activator and a widely reported differentiating agent in various tumors, including glioma cells [36,37] as well as in BEWO cells. Syncytin 1 and 2 mrna expression, the measure of Cell fusion, were stimulated by the presence of forskolin at 24 h at 21% oxygen, which is further abolished at 3% O2. An associated increase in forskolin-induced hCG secretion by BeWo cells, a marker of syncytialisation, was more marked in 21% oxygen (20000 IU/mg protein conc.) compared to 3% oxygen(5000 IU/mg protein conc.). This was in accordance with https://www.sciencedirect.com/science/article/pii/S0925443903000437?via%3Dihub. This was further associated with an increase in progesterone release. The basal levels of estradiol (50000 IU/mg protein), progesterone (140 IU/mg protein) and cortisol (25 IU/mg protein conc.) in BEWO cells were increased at 3% O2. Hypoxia abolishes the increase in B-HCG (5000 IU/mg protein conc.) and progesterone release in BEWO syncytialised cells. No effect on estradiol and cortisol release was seen. Fusion was assessed by co-staining nuclei with phospho and total creb. Confocal microscopy stained with Pcreb Ser133 for 15' at 21% O2 further confirmed BEWO cells were mainly composed of fused cells characterised by a multinucleated syncytium. Cell fusion, as defined by the presence of multinucleated cells, was seldom seen in control cells, but multinucleated cells containing more than four nuclei could always be seen in cell cultures treated with either 100 µM forskolin 24 h. This indicates that forskolin was effective in promoting cytotrophoblast cell differentiation. It was necessary to further determine the effect of crh on trophoblast fusion and the effect of both crh and forskolin under altered oxygen tension.

6.1.8 The effect of CRH on the expression of fusogenic genes in syncytialised bewo cells

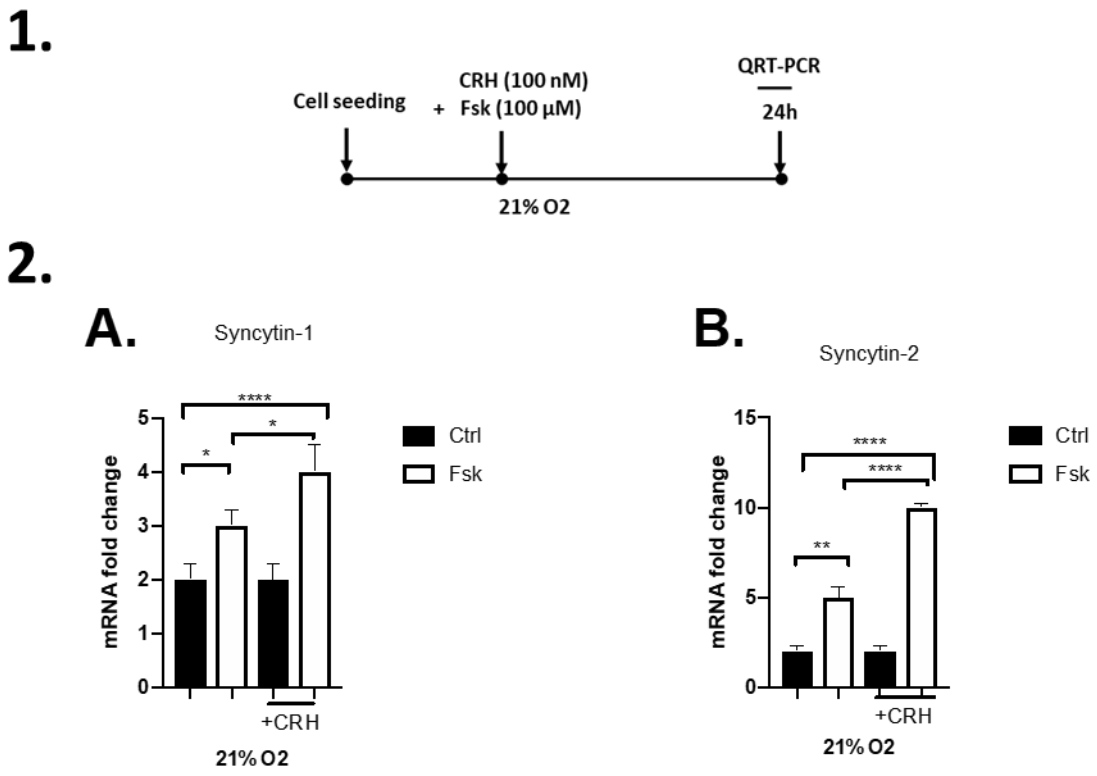


Figure 18

*CRH potentiates the actions of CAMP/PKA signalling pathway on the expression of syncytin 1 and 2 MRNA in BEWO cells. 1. Experiment protocol; where after 80% confluency, BEWO cells are treated with 100nm CRH and 100um forskolin for 24h at 21% O2. 2. QRT PCR analysis showed that CRH potentiated the actions of forskolin on syncytin 1 and 2 in BEWO cells at 24h. CRH alone had no effect on syncytin genes in BEWO cells at 24h. *p< 0.033, **p< .002, ****p< .0001; values are mean +/S.D.; n=3. Data expressed as fold change of gene expression, relative to control; at 24h, after normalising against B-actin. Data were analysed using a one way ANOVA with Tukeys multiple comparison test.*

The effect of CRH on the expression of fusogenic genes in syncytialised bewo cells under altered oxygen tension

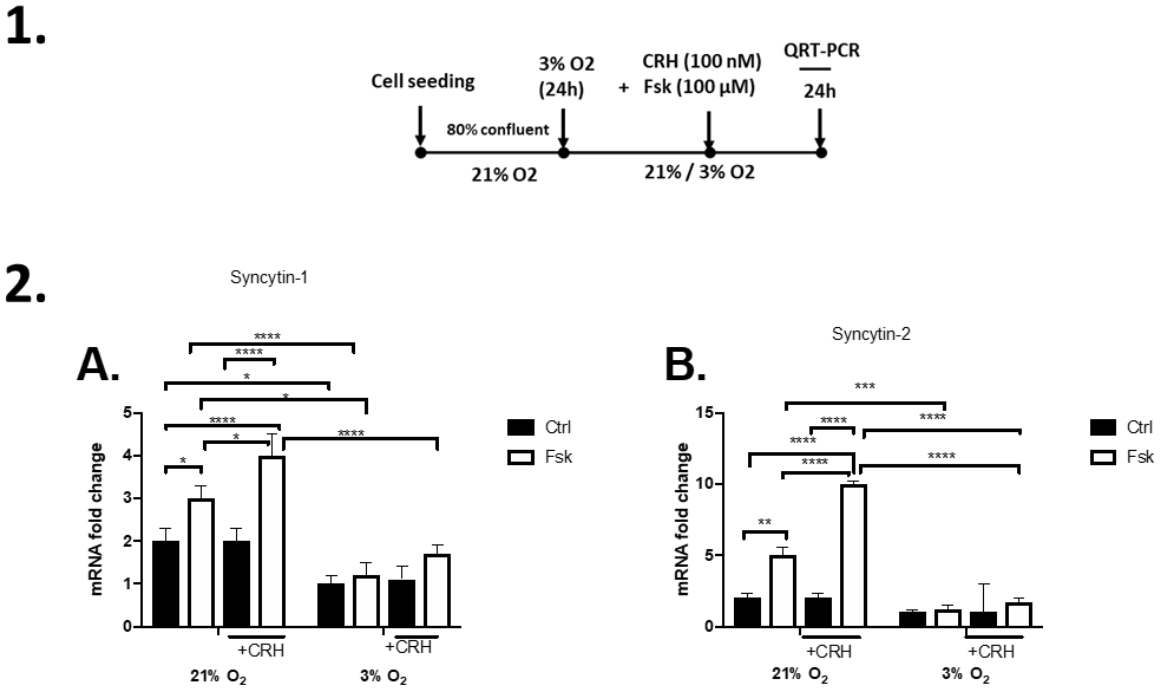


Figure 19

*Hypoxia abolishes the potentiation of syncytin expression by CRH in BEWO syncytialised cells. 1. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were treated with 100nm CRH and 100um forskolin for 24h. 2. QRT PCR analysis showed that under low oxygen tension, CRH was unable to potentiate the actions of forskolin on the MRNA expression of syncytin 1 and 2 in BEWO cells at 24h. CRH had no effect on syncytin genes expression under both high and low oxygen tensions. * p<0.033, ** .002, *** .0002 , ****p< .0001; values are mean +/S.D.; n=3. Data expressed as fold change of gene expression, relative to control at 24h, after normalising against B-actin. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

6.1.9 The effect of CRH on the endocrine capacity and biochemical differentiation in syncytialised BEWO cells

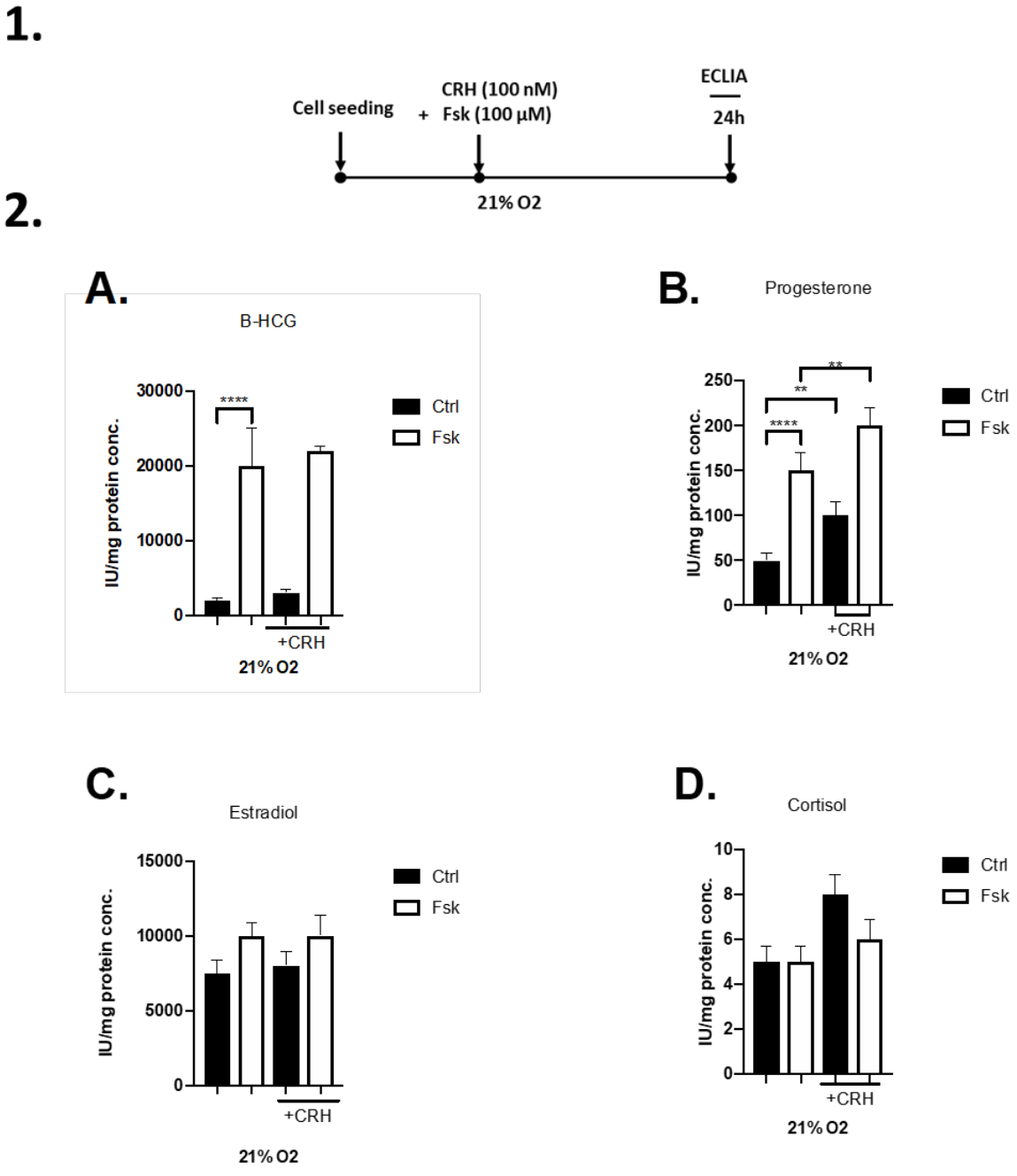


Figure 20

*CRH potentiates the actions of CAMP/PKA signalling pathway on progesterone release, but not B HCG, estradiol and cortisol in BEWO cells. 1. Experiment protocol; where after 80% confluency, BEWO cells are treated with 100nm CRH and 100um forskolin for 24h at 21% O2. 2. ECLIA analysis showed that CRH potentiated the actions of forskolin on progesterone protein release, but not B HCG, estradiol and cortisol release in BEWO cells at 24h. ** .002, ****p< .0001; values are mean +/S.D; n=3. Data expressed as IU/mg protein conc., relative to control 24h, after normalising against B-actin. Data were analysed using a one way ANOVA with Tukeys multiple comparison test.*

The effect of CRH on the endocrine capacity and biochemical differentiation in syncytialised BEWO cells under altered oxygen tension

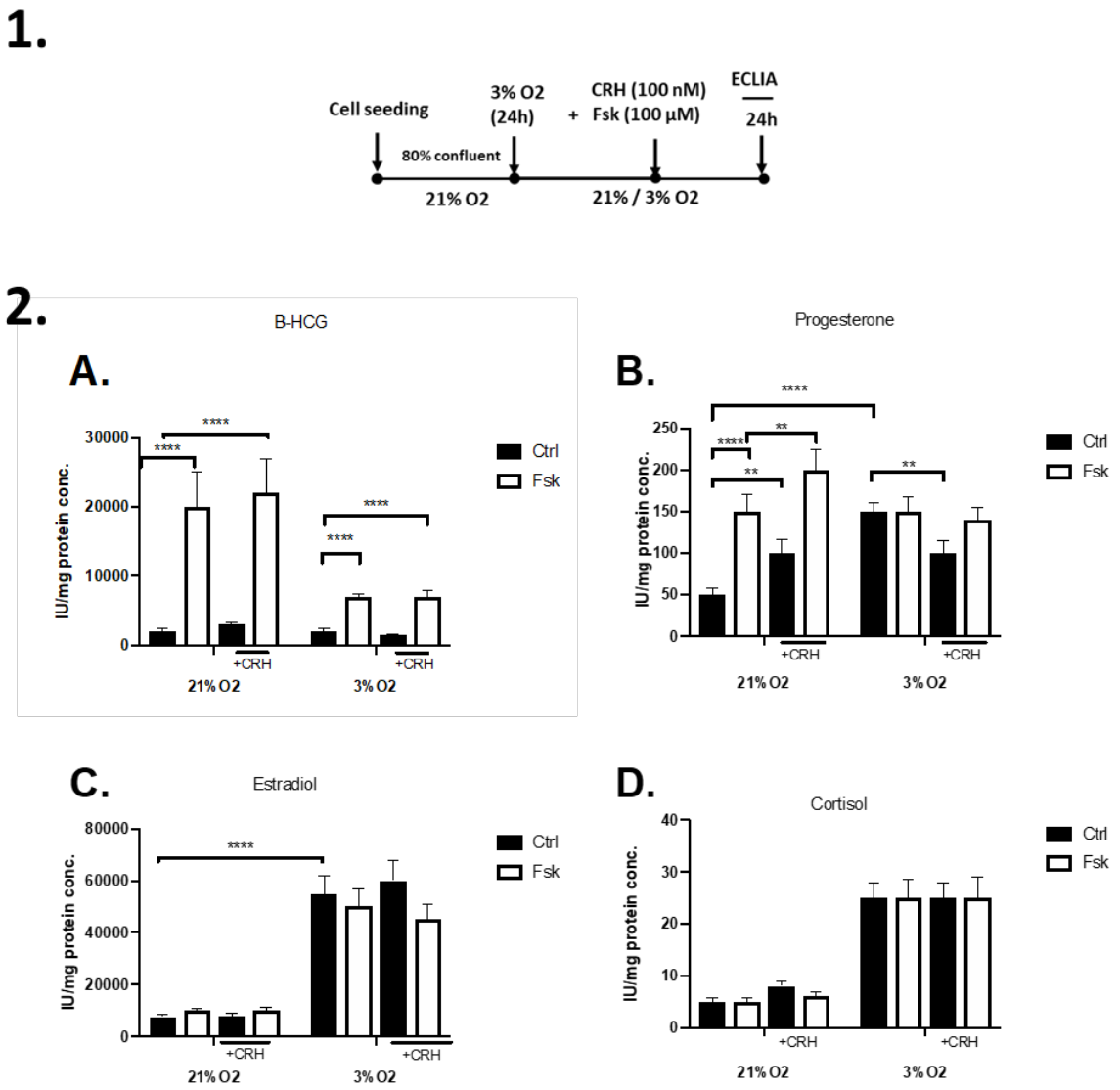


Figure 21

*Under low oxygen tension, CRH reduces progesterone release in BEWO cells and CRH was unable to potentiate the actions of forskolin on progesterone at 24h.Under low oxygen tension, CRH reduces progesterone release in unsyncytialised BEWO cells and the potentiation of progesterone in syncytialised BEWO cells. Hypoxia reversed the actions of CRH on progesterone release in BEWO cells. Hypoxia abolishes the potentiation of progesterone by CRH in BEWO syncytialised cells. Under low oxygen tension, CRH has no effect on B-HCG, estradiol and cortisol release in unsyncytialised and syncytialised BEWO cells. 1. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were treated with 100nm CRH and 100um forskolin for 24h. 2. ECLIA analysis showed that under low oxygen tension, CRH reduces progesterone release in BEWO cells and CRH was unable to potentiate the actions of forskolin on progesterone at 24h. ** .002, ****p< .0001; values are mean +/S.D; n=3. Data expressed as IU/mg protein concentration. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

As camp is a key component of the signalling pathway that promotes cytotrophoblast fusion, it was queried whether crh has any effect on advancing bewo cell differentiation under altered oxygen tension. The effects of forskolin and crh on the molecular markers of

cytotrophoblast differentiation, syncytin mrna expression and hormone secretion (such as B HCG, Estradiol and progesterone) were determined by qrt pcr and elisa assay respectively under both 21 and 3% O2.

CRH had no effect on syncytin 1 and 2 mRNA expression in BEWO cells at 21 % and 3% O2. In BEWO syncytialised cells, under low oxygen tension, the ability of CRH to potentiate the actions of forskolin on syncytin 1 and 2 mRNA expression in BEWO cells was lost. Forksolin and crh increases progesterone release at 21% o2. CRH potentiated the actions of forskolin on progesterone protein release, but not B HCG, estradiol and cortisol release in BEWO cells at 24h. cell differentiation is promoted by both forskolin and CRH. Hypoxia increases progesterone, estradiol and cortisol release in bewo cells.

Under low oxygen tension, CRH has no effect on B-HCG, estradiol and cortisol release in unsyncytialised and syncytialised BEWO cells. Hypoxia reversed the actions of CRH on progesterone release in BEWO cells. Hypoxia abolishes the potentiation of progesterone by CRH in BEWO syncytialised cells. Under low oxygen tension, CRH reduces progesterone release in unsyncytialised BEWO cells and the potentiation of progesterone in syncytialised BEWO cells. Forskolin increases b-hcg release and the effect is reduced under low oxygen tension. Crh potentiates the actions of forskolin on b-hcg release and hypoxia abolishes it. Crh has no effect on b-hcg release under high and low oxygen tension.

6.1.10 The effect of hypoxia on the expression of CRH receptors in BEWO cells

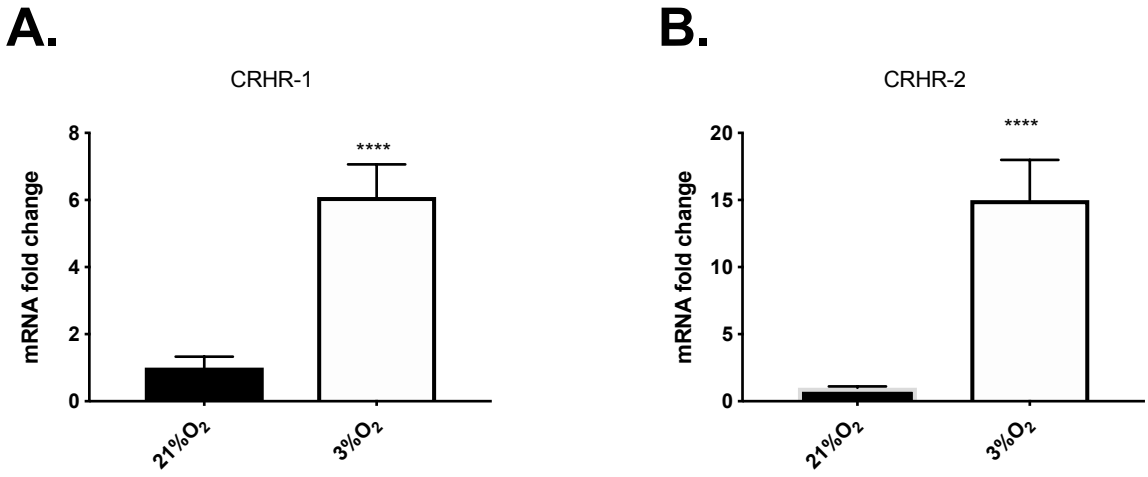


Figure 22

*Hypoxia upregulates the expression of CRHR-1 and 2 MRNA in BEWO cells. QRT PCR analysis showed that the expression of (A) CRHR 1 and (B) CRHR 2 mRNA was significantly upregulated for 24h at 3% O2. *** p=.0002; values are mean +/S.D ; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.*

The effect of CRH on the expression of CRHRs in syncytialised BEWO cells under altered oxygen tension

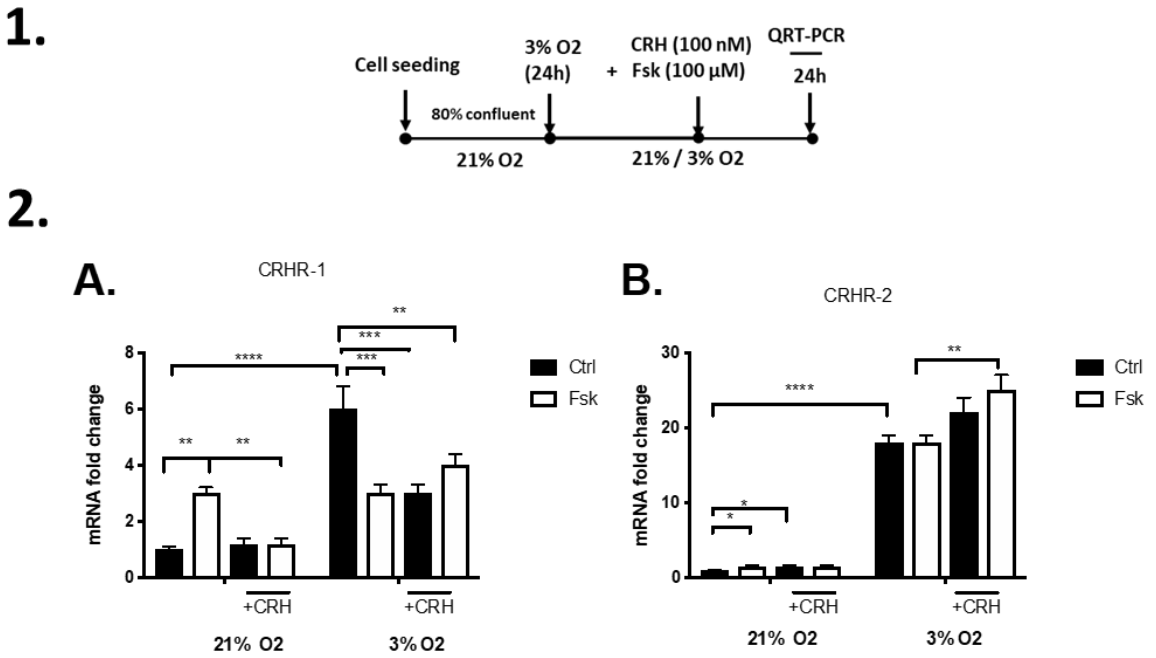


Figure 23

*Hypoxia upregulates CRHR1 and CRHR2 MRNA expression in BEWO cells. CRH potentiates CRHR2 expression in syncytialised BEWO cells. 1. Experimental protocol. 2. QRT PCR analysis showed that Forskolin increases (A) CRHR1 and (B) CRHR2 MRNA expression in BEWO cells. CRH has no effect on CRHR1 but increases CRHR2 mRNA expression in BEWO cells. CRH abolishes the effect of forskolin on MRNA expression of CRHR-1, but not CRHR2 in BEWO cells at 21% O2 at 24h. Hypoxia increases CRHR1 and CRHR2 expression in BEWO cells. Hypoxia reverses the effect of forskolin on CRHR1 expression in BEWO cells. Forskolin has no effect on CRHR2 MRNA expression in BEWO cells. Under low oxygen tension, CRH decreases CRHR1 but not CRHR2 MRNA expression in BEWO cells. Under low oxygen tension, CRH has no effect on CRHR1 expression but potentiates CRHR2 mrna expression in syncytialised BEWO cells. * 0.033, ** .002, *** .0002 , ****p< .0001; values are mean +/S.D; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-way ANOVA with Tukeys multiples comparison test.*

6.1.11 To determine the regulation of CRHRs by CRH and forskolin in BEWO cells under altered oxygen tension

Since crh receptors are crucial in mediating crh bioactivities, lack of potentiation of trophoblast fusion in response to crh under altered oxygen tension prompted us to examine whether the regulation of crhr were being influenced by oxygen tension. Bewo cells were

treated with 100nm crh for 24h and the levels of crhr1 and crhr2 mrna were quantified by qrt pcr. Crh upregulated the expression of crhr2 (2 fold) but not crhr1. This is against the study which showed upregulation of both crhrs in bewo cells by crh .https://rbej.biomedcentral.com/articles/10.1186/1477-7827-11-30#CR24 .Thus indicated that the CRH signalling pathway is intact and that the potential for a positive feed-forward pathway exists in BeWo cells.

It has been previously shown that 8-Br-cAMP increases CRH promoter activity in human primary placental cells [20]. The potential role of CRH on the cAMP-mediated effect on cell survival of BeWo cells under altered oxygen tension needs to be further explored. The effect of camp on crh promoter activity in bewo cells under altered oxygen tension as well needs to be further explored. The effect of forskolin on the expression of endogenous mrna encoding components of crh signalling pathway in bewo cells were examined. Bewo cells were treated with 100um forskolin and the level of crh receptors mrna was quantified by qrt pcr. Mrna levels for crh receptors were stimulated by forskolin. Following exposure to forskolin, the increase of crhr1 and crhr2 mrna was around 3 fold. This is similar to what has been reported earlier https://rbej.biomedcentral.com/articles/10.1186/1477-7827-11-30#CR5. In case mRNA is translated into protein, it may suggest that differentiated BeWo cells are more responsive to CRH cellular action. Hypoxia increases mrna expression of crhr1 (5.5 fold) and crhr2 (15 fold). Under altered oxygen tension, Forskolin reduces crhr1 mrna expression by 50%. The effect seen may be due to basal elevation of crhr1 mrna expression. Hypoxia abolishes the forskolin effect on crhr2 mrna expression.

Discussion

The differentiation of villous cytotrophoblast cells, the main cellular component of the placenta, is critical for a normal pregnancy as they mediate such steps as implantation, pregnancy hormone production, immune protection of the fetus, and delivery [1, 2]. It is known that upon in vitro stimulation with cAMP-inducing agents, such as forskolin, cytotrophoblasts undergo fusion into syncytiotrophoblasts. A syncytiotrophoblast is defined as a giant cell with multiple nuclei sharing one cytoplasm, which expresses certain markers including syncytin 1 and human chorionic gonadotropin (hCG) [3, 4]. The syncytial mass, as assessed by the number of nuclei, increases exponentially across gestation [5]. Many factors

are involved in the process of syncytialisation in the placenta, including cAMP-dependent protein kinase A, various protein tyrosine kinases, protein tyrosine phosphatases, syncytin-1, and GCM1 [4, 6]. While the list of players involved in cytotrophoblast differentiation is rapidly growing, the mechanisms remain far from clear. However, it is remarkable that little is known about the trophoblast cell fusion process (syncytiotrophoblast formation or syncytialisation). Mi et al. [1] suggested that syncytin, a protein encoded by an envelope gene of the recently identified human endogenous retrovirus-W (ERV-W) [2], may mediate placental cytotrophoblast fusion in vivo, and thus be important in human placental morphogenesis. In primary culture of isolated cytotrophoblast cells, the transcript levels of syncytin increase with the differentiation and fusion of cytotrophoblasts into syncytiotrophoblasts [3]. Recent experiments showed that under normoxic conditions (20% O2), relative mRNA abundance for syncytin is enhanced and for its receptor, amino acid transport system B0 (ASCT2), is suppressed in BeWo cells during syncytialisation by forskolin [6]. It has been reported that placental syncytin mRNA expression level is reduced in pregnancies complicated with pre-eclampsia [7] and its extreme form, hemolysis, elevated liver enzymes and low platelets syndrome [8]. The histological abnormalities of placentae in pregnancy associated with underperfusion and hypoxia are characterised by cytotrophoblast prominence and abnormalities in syncytiotrophoblast formation [10]. It also has been demonstrated that trophoblast cell fusion and differentiation are inhibited by hypoxia [11], [12], [13]. These observations have raised the possibility that low oxygen may, via regulation of syncytin gene expression and associated hormones such as B-HCG, suppress trophoblast cell fusion.

Corticotrophin releasing hormone (CRH), one of the hypothalamic “stress” peptides, plays a pivotal role in mammalian survival and adaptation responses involving the activation of the hypothalamic pituitary adrenal (HPA) axis. Besides its presence in the central nervous system, CRH is also expressed in the human placenta [7]. Placental CRH becomes detectable in maternal plasma around 16~20 weeks gestation, and increases exponentially as pregnancy progresses towards term. The level of placental CRH in maternal circulation has been linked to the length of gestation [8, 9, 10]. Placental CRH appears to target multiple feto-maternal tissues, including the foetal adrenals, myometrial smooth muscle, placenta, placental vasculature and foetal membranes [11, 12, 13]. Through the various CRH receptor subtypes (CRHR1 and CRHR2) CRH plays diverse roles at different stages of pregnancy and labour. For

example, CRH stimulates the foetal pituitary-adrenal axis, modulates placental vascular tone and endocrine function (especially prostaglandin generation), controls myometrial contractility ⁄ quiescence, and regulates trophoblast cell growth and invasion [10]. Nevertheless, there is little research literature available on a role for CRH in trophoblast cell differentiation.

Stimulation by 8-Br-cAMP of BeWo cells cultured in normal foetal calf serum resulted in higher levels of hCG and ERVW-1 [14]. The effect of 8-Br-cAMP on the expression of endogenous mRNA encoding components of the CRH signalling pathway was shown in BeWo cells. CRH promoter activity is increased by 8-Br-cAMP in human placental cells [15], while others have shown that CRH can act via CRH-receptors to increase cAMP production [16, 17]. Therefore, the potential roles of CRH on the differentiation of human trophoblast BeWo cells under altered oxygen tension were investigated.

It was found that under low oxygen tension, syncytin 1 and 2 mrna expression, B –HCG release is reduced in bewo syncytialised cells. It is not known if the effects can be reversed and needs to be further investigated. The progesterone release is abolished. Estradiol and cortisol release remains unaffected. This is associated with downregulation of CRHR1 and inability of CRHR2 upregulation. Further investigation on syncytin proteins and its receptors, amino acid transport system B are necessary to be established which are thought to be involved in trophoblast cell fusion (as well as the activity of amino acid transport through this system) in a cell model of syncytialisation (BeWo cells following forskolin treatment). Hypoxia increases progesterone, estradiol and cortisol release except B-HCG release. This is further associated with CRHR2 mRNA being more upregulated compared to CRHR1. These observations suggest that under conditions of low ambient oxygen, dysregulation of expression of syncytin and hormones may suppress the normal process of cell fusion necessary for syncytiotrophoblast formation and contributes to syncytiotrophoblast abnormalities characteristic of pre-eclampsia. This may perhaps be mediated via downregulation of crhr1 expression and loss of crhr2 upregulation.

CRH is known to activate the cAMP/PKA (protein kinase A) pathway via its receptors, CRHR1 and CRHR2, leading to phosphorylation of the cAMP response element binding protein

(CREB1) [25]. As a transcription factor, CREB1 binds to a cAMP response element (CRE) sequence in the promoter of target genes, regulating the transcription of those genes [26, 27]. CRH has been studied as one of those target genes containing a CRE in the promoter, and the CRE has been shown to be crucial for CRH gene expression [28, 29, 30]. Furthermore, CRH and its receptors (CRHR1/R2) are expressed in placental tissues [9, 31] and CRH can bind to syncytiotrophoblast membranes [32]. So, the induction of CRH by cAMP hints that CRH may play a feedback role in cAMP mediated effects in placental trophoblasts.

Under high oxygen tension, CRH potentiates syncytins mRNA and progesterone release in syncytialised BEWO cells. CRHR1 and CRHR2 expression remains unaffected. The CRH effect on syncytins genes and progesterone release is lost under low oxygen tension. This is further associated with CRH having no effect on the downregulated CRHR1 expression in BEWO syncytialised cells but upregulates CRHR2 expression.

Since syncytialization was dysregulated if cells were cultured under altered oxygen tension, it led us to imply that CRH signal pathways may play a role in cAMP-mediated cell apoptosis and differentiation. The ability of CRH to regulate cell apoptosis in several cells of different origin has been reported [33, 34], and CRH has been shown to induce FASLG (Fas ligand) production and apoptosis in the rat pheochromocytoma cell line PC12 via activation of MAPK (p38 mitogen-activated protein kinase) [22]. It can thus be further proposed that although CRH is produced by differentiated trophoblasts it may also play a role in regulating trophoblast viability, thereby helping to keep the syncytiotrophoblast pool in renewal and this may be altered under low oxygen tension. Nevertheless, the existence of CRH receptors in BeWo cells suggests that CRH could be involved in another bioactivity, such as modulation of differentiation or syncytialisation of trophoblast cells. Consistent with previously published data, it was demonstrated that CRH can induce cell differentiation, as evidenced by the induction of ERVW-1 and by cell fusion. For trophoblast cells, the widely accepted marker of differentiation is a combination of a biochemical index (induction of hCG and ERVW-1 proteins) and a morphological index (formation of cell fusion), with the latter being more convincing. Recently, the reliability of hCG as a trophoblast differentiation marker has been called into question, due to dissociation of the two differentiation indexes. Similar to what has been published, It was shown here additional evidence that biochemical and

morphological differentiation can be dissociated, since CRH facilitates cell fusion without any induction of hCG secretion. Also, CRH stimulation induced ERVW-1 expression, without any induction of hCG secretion [35].

Similar dissociations have been reported in villous cytotrophoblast cells cultured in the absence of serum [36] and in JEG3 cells stimulated by forskolin, where an induction of hCG occurred without any evidence of cell fusion [21]. Recently, it was reported that the PKA inhibitor, H89, can reverse forskolin-induced BeWo cell fusion without altering forskolin-induced hCG secretion [37]. Therefore, hCG protein expression may not necessarily be linked to syncytial fusion. This study adds to the growing evidence that differentiation and fusion are related but distinct events [38]. However, the secretion of hCG can be enhanced by 8-Br-cAMP but not by CRH, suggesting that CRH and 8-Br-cAMP may regulate cell fusion through different signal pathways. Indeed, others have shown that charcoal scavenged hormones, such as estradiol, glucocorticoids and hCG, play roles in trophoblast differentiation at different stages of pregnancy [39].

That the expression of CRHR1 and CRHR2 can be up regulated by 8-Br-cAMP and also by CRH itself. There may exist a positive feed-forward mechanism in the CRH/CRHR/cAMP/PKA/CREB1 signal pathway, and that this occurs at several different levels. The positive communication existing with CRH and its receptors has the potential to magnify CRH bioactivities by, for example, promotion of trophoblast cell syncytialization. Since placental CRH expression in trophoblast cells begins when the cells differentiate toward syncytialization, the positive feed-forward and promotion of syncytialization by CRH could explain the exponential increase of CRH detected in maternal plasma as pregnancy progresses towards term, particularly as syncytial nuclear numbers increase exponentially across gestation [5]. This may provide the mechanism that links maturation of the placental structure to the timing of birth.

Table 15

	21%O2				3% O2			
	-Fsk		+Fsk		-Fsk		+Fsk	
	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH
Syncytin 1	NS	NS	↑	↑	↑	NS	NS	NS
Syncytin 2	NS	NS	↑	↑	↑	NS	NS	NS
B-HCG	NS	NS	↑	NS	NS	NS	↑	NS
Estradiol	NS	NS	NS	NS	NS	NS	NS	NS
Progesterone	NS	↑	↑	↑	↑	↓	NS	NS
Cortisol	NS	NS	NS	NS	↑	NS	NS	NS
CRHR-1	NS	NS	↑	↓	↑	↓	↓	NS
CRHR-2	NS	↑	↑	NS	↑	NS	NS	↑

Table 16

	Syncytin-1		Syncytin-2		B-HCG		Estradiol		Progesterone		Cortisol		CRHR1		CRHR2	
	21% O2	3 % O 2	21% O2	3 % O 2	21 % O2	3 % O 2	21% O2	3 % O 2	21% O2	3 % O 2	21% O2	3 % O 2	21% O2	3 % O 2	21% O2	3 % O 2

Ctrl (Fsk)	NS	↑	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	↓	NS	↓
CRH (Fsk)	↑	NS	NS	NS	↑	NS	NS	NS	↑	NS	NS	NS	↑	NS	↑	NS
CRH (CRH + Fsk)	↑	NS	NS	↑	NS	NS	NS	NS	↑	NS	NS	NS	↓	NS	↑	NS

6.1.12 The effect of CRH receptor activation on fusogenic machinery in syncytialised BEWO cells

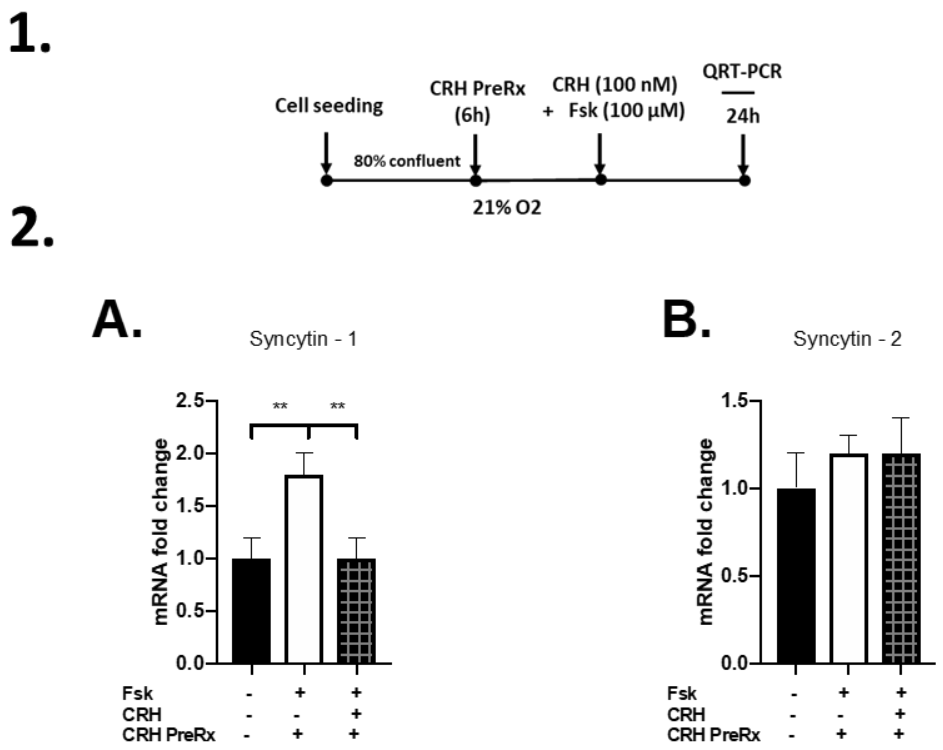


Figure 24

*priming BEWO cells with CRH potentiates the actions of CAMP signalling pathway on the expression of syncytin 1 but not syncytin 2 at 21% O2. 1. Experimental protocol, where cells were primed with 100nM CRH for 6h for activating CRHRs. This was further followed by treating BEWO cells with CRH and forskolin for 24h and MRNA expression was investigated by QRT-PCR. 2. QRT PCR analysis showed pretreating cells with CRH showed increased mrna expression of (A) syncytin1 but not (B) syncytin2 in bewo syncytialised cells. prolonged treatment with crh further showed loss of enhanced mrna expression of syncytin1. No change in the expression of syncytin 2 was seen. **p= .002; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a one-way ANOVA with Tukeys multiples comparison test.*

Having established that CRH was unable to restore impaired differentiation in trophoblasts, it was further investigated whether priming cells with CRH, there by activating CRH receptors before differentiation of trophoblasts would influence syncytialization. Furthermore, it was assessed whether this mechanism was influenced by altered oxygen tension. This alteration in experimental set up was done to rule out the possibility that CRH forskolin interactions may interfere with CRH receptor expression and perhaps thus CRH is not able to act on its receptors. Also, the role of CRH on activation of its receptors in differentiated BEWO cells as well as the influence of oxygen tension needs to be investigated. So as to test this hypothesis, BEWO cells were pre-treated with 100nM CRH for 6 hours. Supernatants were removed, and cells were further incubated with 100 nM CRH and 100 uM forskolin for 24h, under low and high oxygen tension. mrna expression of syncytin 1, 2; CRHR1 and CRHR2 were measured using QRT -PCR and the release of B-HCG, estradiol, progesterone and cortisol were measured by ECLIA. QRT PCR analysis showed pretreating cells with CRH showed increased mrna expression of (A) syncytin1 but not (B) syncytin2 in bewo syncytialised cells. prolonged treatment with crh further showed loss of enhanced mrna expression of syncytin1. No change in the expression of syncytin 2 was seen. hypoxia increased syncytin 1 but not syncytin 2 mrna expression. Hypoxia abolished the priming effect of crh on syncytin 1 expression, but potentiated the effect of extended CRH treatment on syncytin 2 expression in syncytialised bewo cells. priming BEWO cells with CRH potentiates the actions of CAMP signalling pathway on the release of B-HCG (25000 IU/mg protein conc.) at 21% O2. Further extended treatment with CRH showed potentiation of actions of CAMP signalling pathway on the release of progesterone (175 IU/mg conc.). No effect on estradiol release is seen.

QRT-PCR analysis showed priming BEWO cells with CRH potentiate the actions of CAMP signalling pathway on the expression of CRHR1 and CRHR2 at 21% O2. Extended treatment with CRH potentiates CRHR2 expression but abolishes the potentiation of CRHR1 expression. ECLIA analysis showed priming cells with crh potentiated actions of forskolin on B-HCG and progesterone release in BEWO cells. Extended CRH treatment further potentiates progesterone release in BEWO cells at 21% O2. Hypoxia abolishes the effect of CRH priming on forskolin mediated B-HCG and progesterone release in BEWO cells.

QRT PCR analysis showed Priming cells with CRH upregulates CRHR1 and CRHR2 mRNA expression in syncytialised bewo cells. Extended CRH treatment further potentiates the actions of forskolin on CRHR2 mrna expression at 21% O2. low oxygen tension reduces CRHR1 and 2 mrna expression and abolishes the priming effect of crh on CRHR1 and 2 mRNA expression in syncytialised bewo cells.

Syncytin 1 and 2 mrna expression, the measure of Cell fusion, were stimulated by the presence of forskolin at 24 h at 21% oxygen, which is further abolished at 3% O2. An associated increase in forskolin-induced hCG secretion by BeWo cells, a marker of syncytialisation, was more marked in 21% oxygen (20000 IU/mg protein conc.) compared to 3% oxygen(5000 IU/mg protein conc.). This was in accordance with https://www.sciencedirect.com/science/article/pii/S0925443903000437?via%3Dihub. This was further associated with an increase in progesterone release. The basal levels of estradiol (50000 IU/mg protein), progesterone (140 IU/mg protein) and cortisol (25 IU/mg protein conc.) in BEWO cells were increased at 3% O2. Hypoxia abolishes the increase in B-HCG (5000 IU/mg protein conc.) and progesterone release in BEWO syncytialised cells. No effect on estradiol and cortisol release was seen. This indicates that forskolin was effective in promoting cytotrophoblast cell differentiation. It was necessary to further determine the effect of crh on trophoblast fusion and the effect of both crh and forskolin under altered oxygen tension.

6.1.13 The effect of CRH receptor activation on biochemical differentiation and endocrine capacity in syncytialised BEWO cells

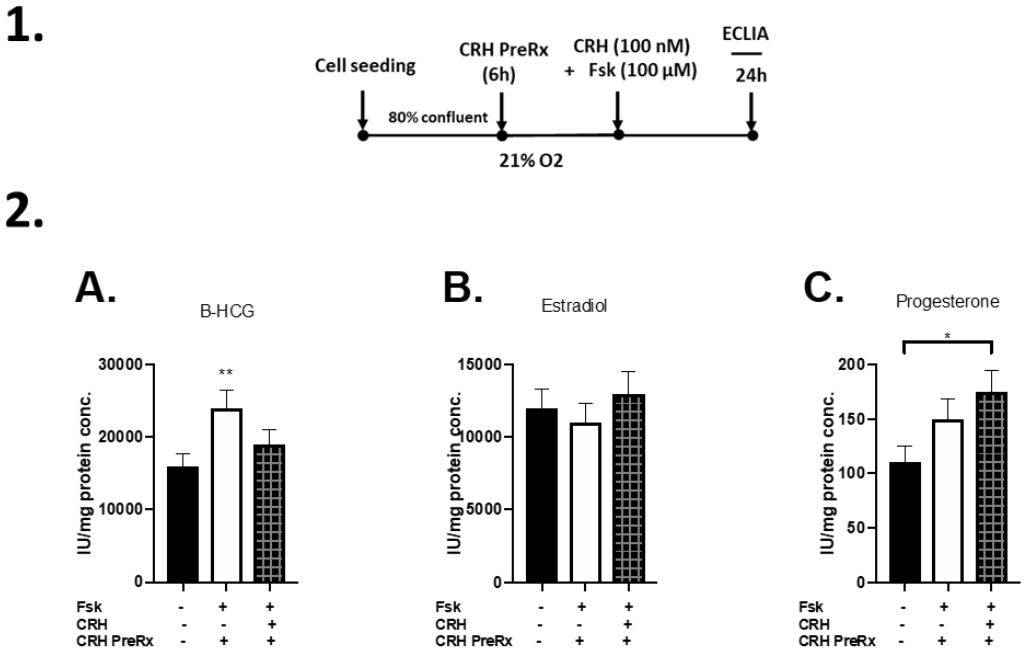


Figure 25

*priming BEWO cells with CRH potentiates the actions of CAMP signalling pathway on the release of B-HCG at 21% O2. 1. Experimental protocol, where cells were primed with 100nM CRH for 6h for activating CRHRs. This was further followed by treating BEWO cells with CRH and forskolin for 24h and protein release was investigated by ECLIA. 2. ECLIA analysis showed priming BEWO cells with CRH potentiates the actions of CAMP signalling pathway on the release of B-HCG at 21% O2. Further extended treatment with CRH showed potentiation of actions of CAMP signalling pathway on the release of progesterone. No effect on estradiol release is seen. * 0.033, **p= .002; n=3. Data expressed as IU/mg protein concentration. Data were analysed using a one-way ANOVA with Tukeys multiples comparison test.*

As camp is a key component of the signalling pathway that promotes cytotrophoblast fusion, it was queried whether crh has any effect on advancing bewo cell differentiation under altered oxygen tension. The effects of forskolin and crh on the molecular markers of cytotrophoblast differentiation, syncytin mrna expression and hormone secretion (such as B HCG, Estradiol and progesterone) were determined by qrt pcr and elisa assay respectively under both 21 and 3% O2.

CRH had no effect on syncytin 1 and 2 mRNA expression in BEWO cells at 21 % and 3% O2. In BEWO syncytialised cells, under low oxygen tension, the ability of CRH to potentiate the actions of forskolin on syncytin 1 and 2 mRNA expression in BEWO cells was lost. Forksolin and crh increases progesterone release at 21% o2. CRH potentiated the actions of forskolin on progesterone protein release, but not B HCG, estradiol and cortisol release in BEWO cells

at 24h. cell differentiation is promoted by both forskolin and CRH. Hypoxia increases progesterone, estradiol and cortisol release in bewo cells.

Under low oxygen tension, CRH has no effect on B-HCG, estradiol and cortisol release in unsyncytialised and syncytialised BEWO cells. Hypoxia reversed the actions of CRH on progesterone release in BEWO cells. Hypoxia abolishes the potentiation of progesterone by CRH in BEWO syncytialised cells. Under low oxygen tension, CRH reduces progesterone release in unsyncytialised BEWO cells and the potentiation of progesterone in syncytialised BEWO cells. Forskolin increases b-hcg release and the effect is reduced under low oxygen tension. Crh potentiates the actions of forskolin on b-hcg release and hypoxia abolishes it. Crh has no effect on b-hcg release under high and low oxygen tension.

6.1.14 The effect of CRH receptor activation on the expression of CRH receptors in syncytialised BEWO cells

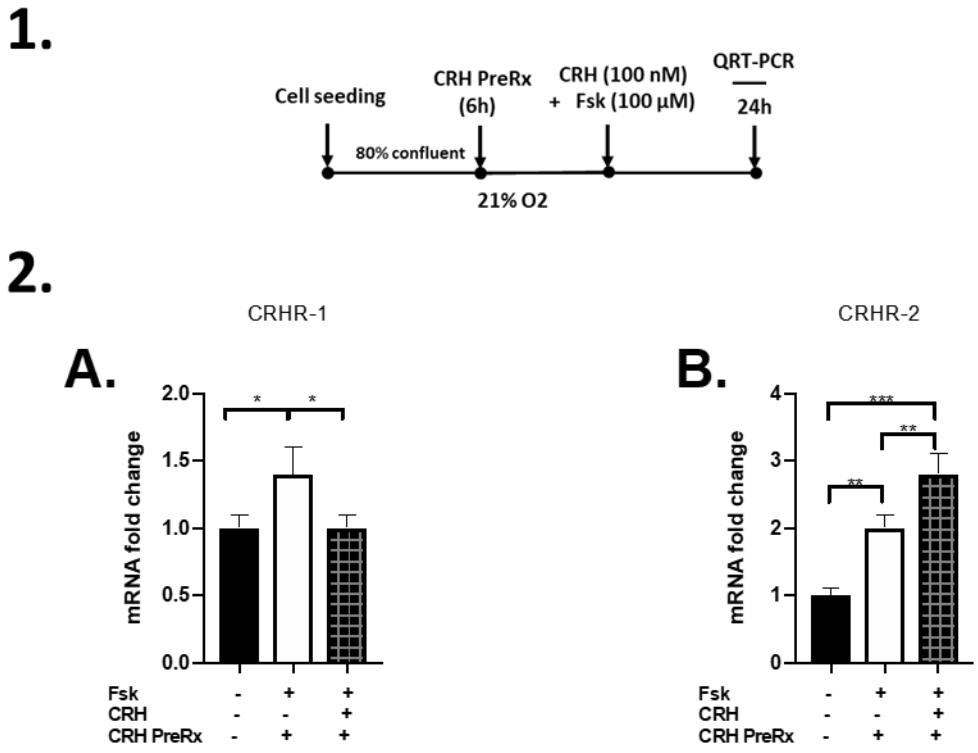


Figure 26

*priming synyctialised BEWO cells with crh upregulates CRHR1 and CRHR2 mrna expression. 1. Experimental protocol, where cells were primed with 100nM CRH for 6h for activating CRHRs. This was further followed by treating BEWO cells with CRH and forskolin for 24h and MRNA fold change was investigated by QRT-PCR. 2. QRT-PCR analysis showed priming BEWO cells with CRH potentiate the actions of CAMP signalling pathway on the expression of CRHR1 and CRHR2 at 21% O2. Extended treatment with CRH potentiates CRHR2 expression but abolishes the potentiation of CRHR1 expression. p= * 0.033, ** .002; *** .0002, n=3. Data expressed as IU/mg protein concentration. Data were analysed using a one-way ANOVA with Tukeys multiples comparison test.*

Since crh receptors are crucial in mediating crh bioactivities, lack of potentiation of trophoblast fusion in response to crh under altered oxygen tension prompted us to examine whether the regulation of crhr were being influenced by oxygen tension. Bewo cells were treated with 100nm crh for 24h and the levels of crhr1 and crhr2 mrna were quantified by qrt pcr. Crh upregulated the expression of crhr2 (2 fold) but not crhr1. This is against the study which showed upregulation of both crhrs in bewo cells by crh .https://rbej.biomedcentral.com/articles/10.1186/1477-7827-11-30#CR24 .Thus indicated that the CRH signalling pathway is intact and that the potential for a positive feed-forward pathway exists in BeWo cells.

6.1.15 The effect of CRH receptor activation on fusogenic machinery in syncytialised BEWO cells under altered oxygen tension

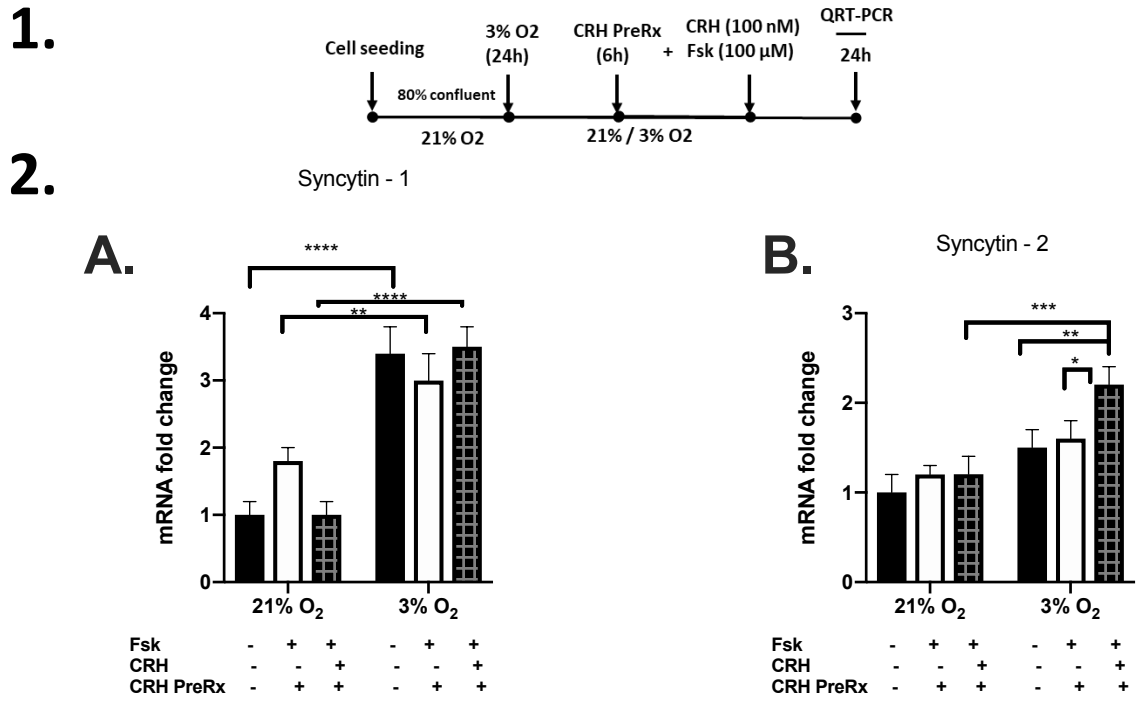


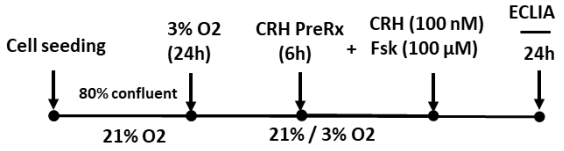
Figure 27

*Low oxygen tension upregulates syncytin1 mrna expression and abolishes the priming effect of crh on syncytin 1 expression in syncytialised bewo cells. 1. Experimental protocol, where cells were incubated at 3% O2 for 24h, following which cells were primed with 100nM CRH for 6h for activating CRHRs. This was further followed by treating BEWO cells with CRH and forskolin for 24h and MRNA expression was investigated by QRT-PCR. 2. QRT PCR analysis showed hypoxia increases syncytin 1 but not syncytin 2 mrna expression. Hypoxia abolishes the priming effect of crh on syncytin 1 expression, but potentiates the effect of extended CRH treatment on syncytin 2 expression in syncytialised bewo cells. p= * 0.033, ** .002; *** .0002; ****p< .0001; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-way ANOVA with Tukeys multiples comparison test.*

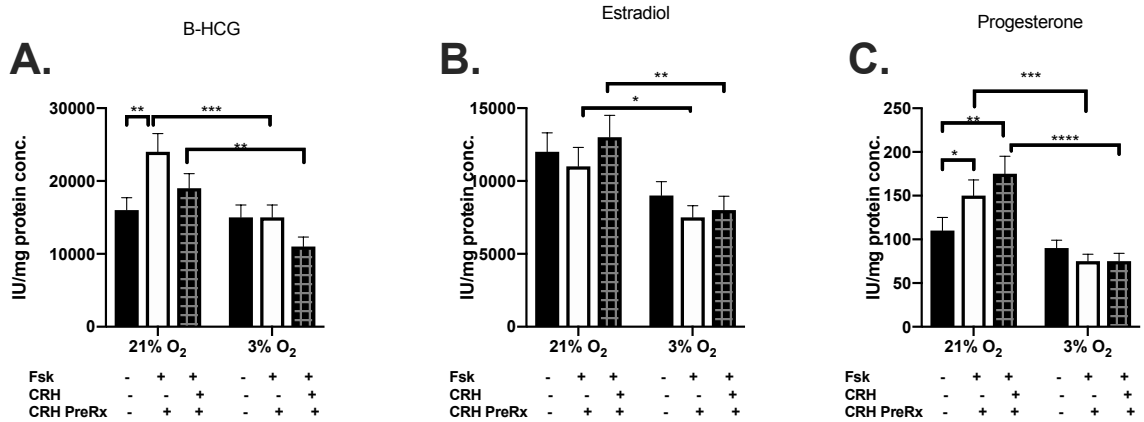
It has been previously shown that 8-Br-cAMP increases CRH promoter activity in human primary placental cells [20]. The potential role of CRH on the cAMP-mediated effect on cell survival of BeWo cells under altered oxygen tension needs to be further explored. The effect of camp on crh promoter activity in bewo cells under altered oxygen tension as well needs to be further explored. The effect of forskolin on the expression of endogenous mrna encoding components of crh signalling pathway in bewo cells were examined. Bewo cells were treated with 100um forskolin and the level of crh receptors mrna was quantified by qrt pcr. Mrna levels for crh receptors were stimulated by forskolin. Following exposure to forskolin, the increase of crhr1 and crhr2 mrna was around 3 fold. This is similar to what has been reported earlier https://rbej.biomedcentral.com/articles/10.1186/1477-7827-11-30#CR5. In case mRNA is translated into protein, it may suggest that differentiated BeWo cells are more responsive to CRH cellular action. Hypoxia increases mrna expression of crhr1 (5.5 fold) and crhr2 (15 fold). Under altered oxygen tension, Forskolin reduces crhr1 mrna expression by 50%. The effect seen may be due to basal elevation of crhr1 mrna expression. Hypoxia abolishes the forskolin effect on crhr2 mrna expression.

6.1.16 The effect of CRH receptor activation on biochemical differentiation and endocrine capacity in syncytialised BEWO cells under altered oxygen tension

1.



2.



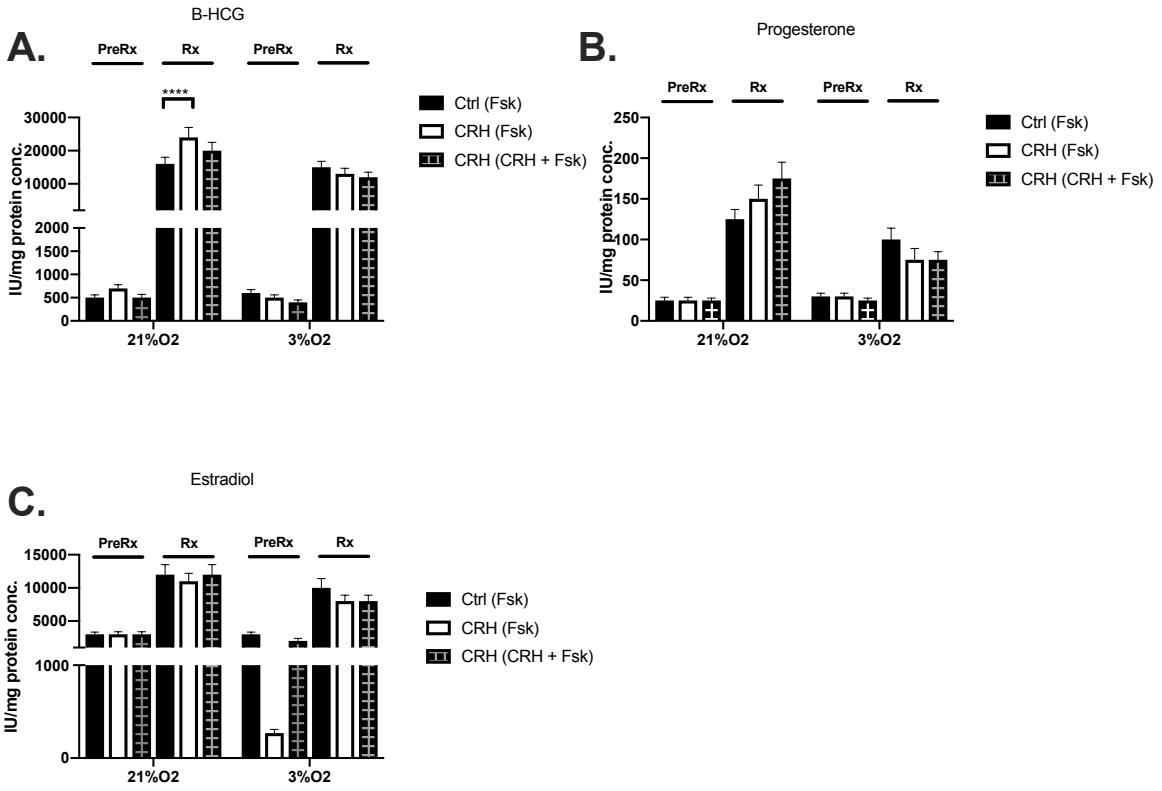


Figure 28

*Hypoxia abolishes the effect of CRH priming on forskolin mediated B-HCG and progesterone release in BEWO cells. 1. Experimental protocol, where cells were incubated at 3% O2 for 24h, following which cells were primed with 100nM CRH for 6h for activating CRHRs. This was further followed by treating BEWO cells with CRH and forskolin for 24h and protein release was investigated by ECLIA. 2. ECLIA analysis showed priming cells with crh potentiated actions of forskolin on B-HCG and progesterone release in BEWO cells. Extended CRH treatment further potentiates progesterone release in BEWO cells at 21% O2. Hypoxia abolishes the effect of CRH priming on forskolin mediated B-HCG and progesterone release in BEWO cells. p= * 0.033, ** .002; *** .0002; ****p< .0001 n=3. Data expressed as IU/mg protein concentration. Data were analysed using a two-way ANOVA with Tukeys multiples comparison test.*

6.1.17 The effect of CRH receptor activation on the expression of CRH receptors in syncytialised BEWO cells under altered oxygen tension

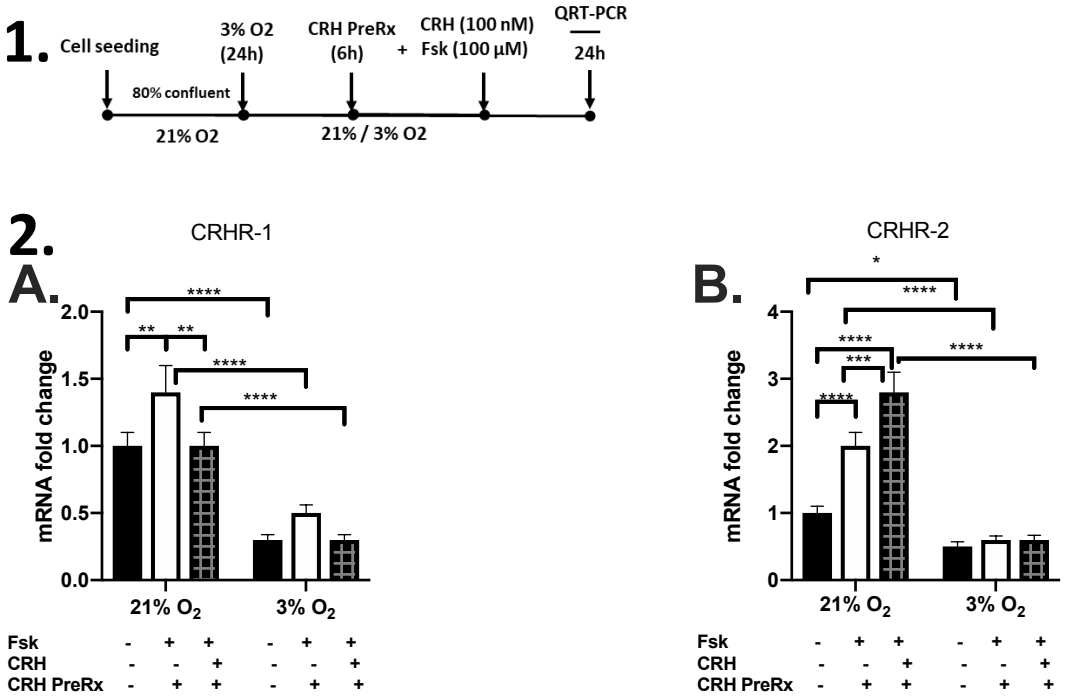
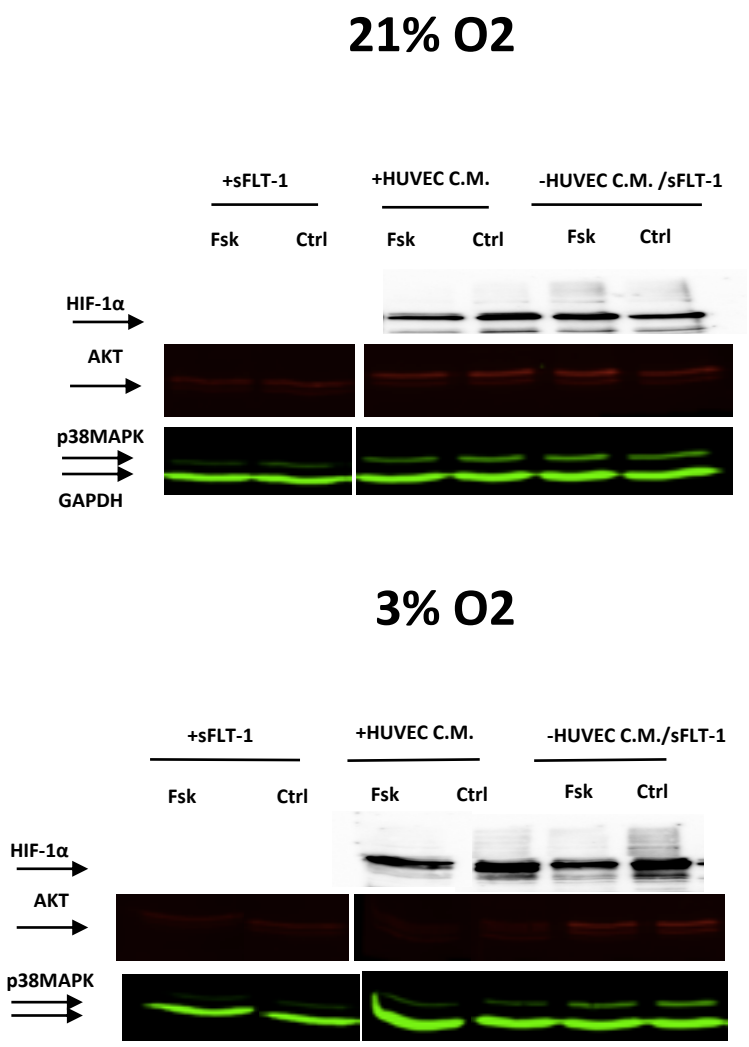


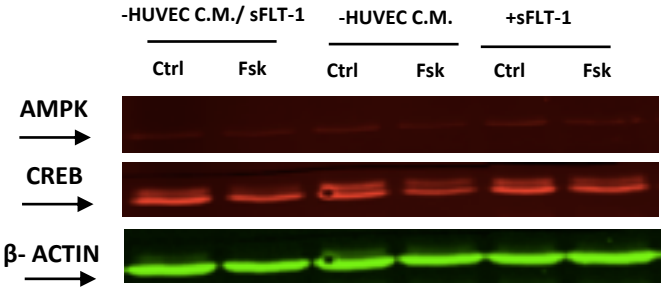
Figure 29

*Hypoxia abolishes the effect of priming of cells with CRH on CRHRs expression in syncytialised bewo cells. 1.Experimental protocol, where cells were incubated at 3% O2 for 24h, following which cells were primed with 100nM CRH for 6h for activating CRHRs. This was further followed by treating BEWO cells with CRH and forskolin for 24h and MRNA expression was investigated by QRT-PCR. 2. QRT PCR analysis showed Priming cells with CRH upregulates CRHR1 and CRHR2 mRNA expression in syncytialised bewo cells. Extended CRH treatment further potentiates the actions of forskolin on CRHR2 mrna expression at 21% O2. low oxygen tension reduces CRHR1 and 2 mrna expression and abolishes the priming effect of crh on CRHR1 and 2 mRNA expression in syncytialised bewo cells. p= * 0.033, ** .002; *** .0002; ****p< .0001; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-way ANOVA with Tukeys multiples comparison test.*

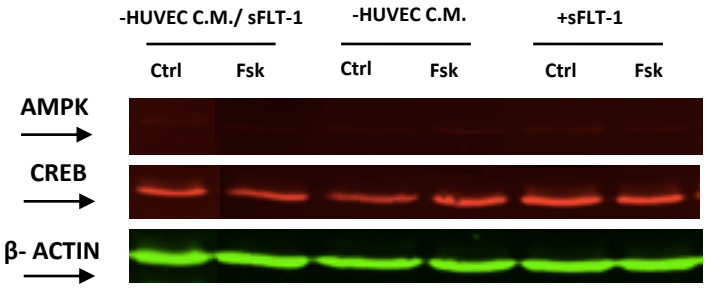
6.1.18 Characterization of signalling molecules in BEWO cells in response to camp agonist



21% O2


-HUVEC C.M./ sFLT-1
-HUVEC C.M.
+sFLT-1
Ctrl
Fsk
Ctrl
Fsk
Ctrl
Fsk
AMPK
CREB
β- ACTIN

3% O2


-HUVEC C.M./ sFLT-1
-HUVEC C.M.
+sFLT-1
Ctrl
Fsk
Ctrl
Fsk
Ctrl
Fsk
AMPK
CREB
β- ACTIN

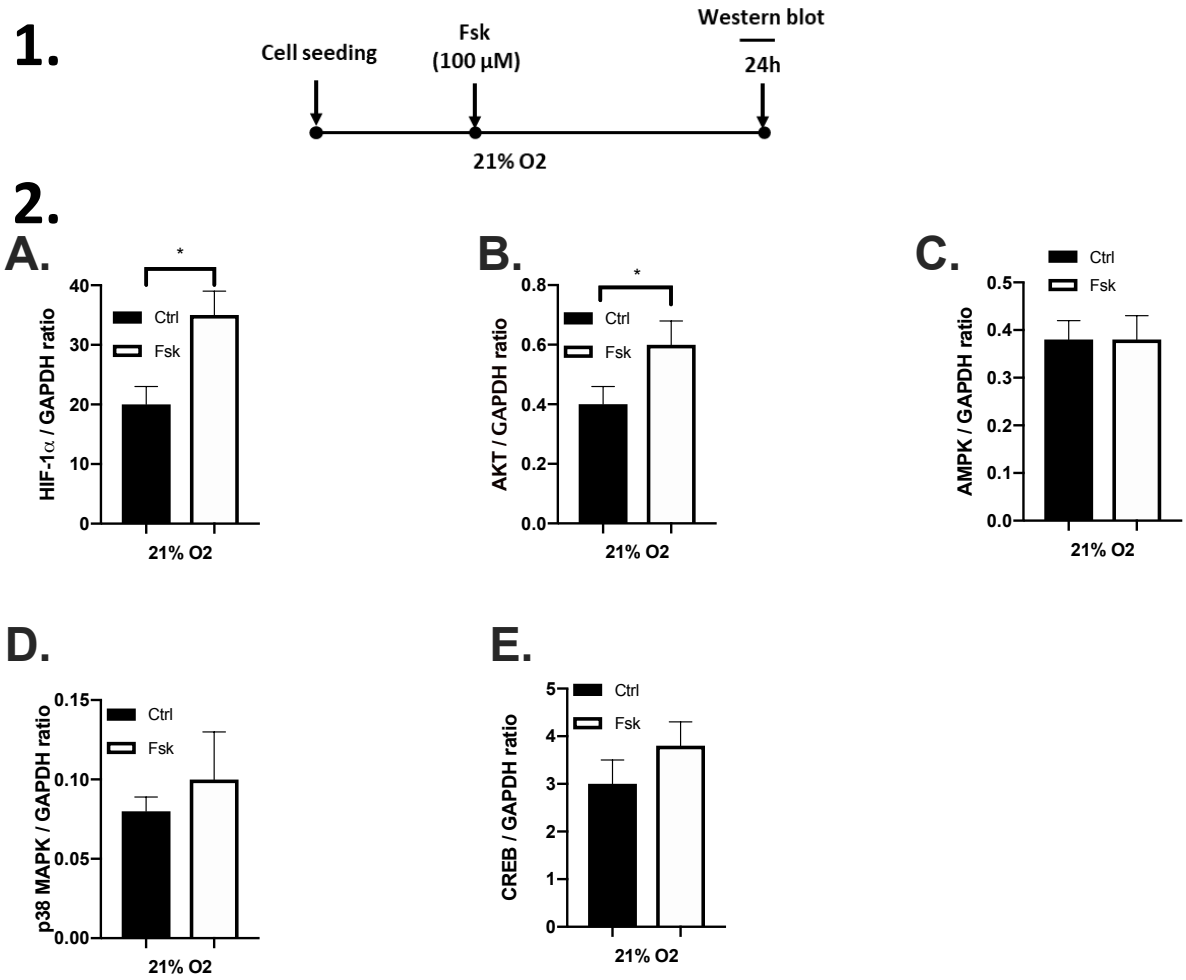


Figure 30

*Under high oxygen tension (21%O2), Forskolin (Fsk) increases HIF-1a and AKT protein expression. 1. Experimental protocol; 2. Western blot analysis showed treating cells with 100um forskolin for 24h showed increased protein expression of HIF-1a, AKT but not AMPK, P38 MAPK and CREB. p= * 0.033, n=3. GAPDH was used as a loading control in each sample. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against GAPDH. Data were analysed using a two tailed unpaired t test.*

6.1.19 Characterization of signalling molecules in BEWO cells under altered oxygen tension in response to camp agonist

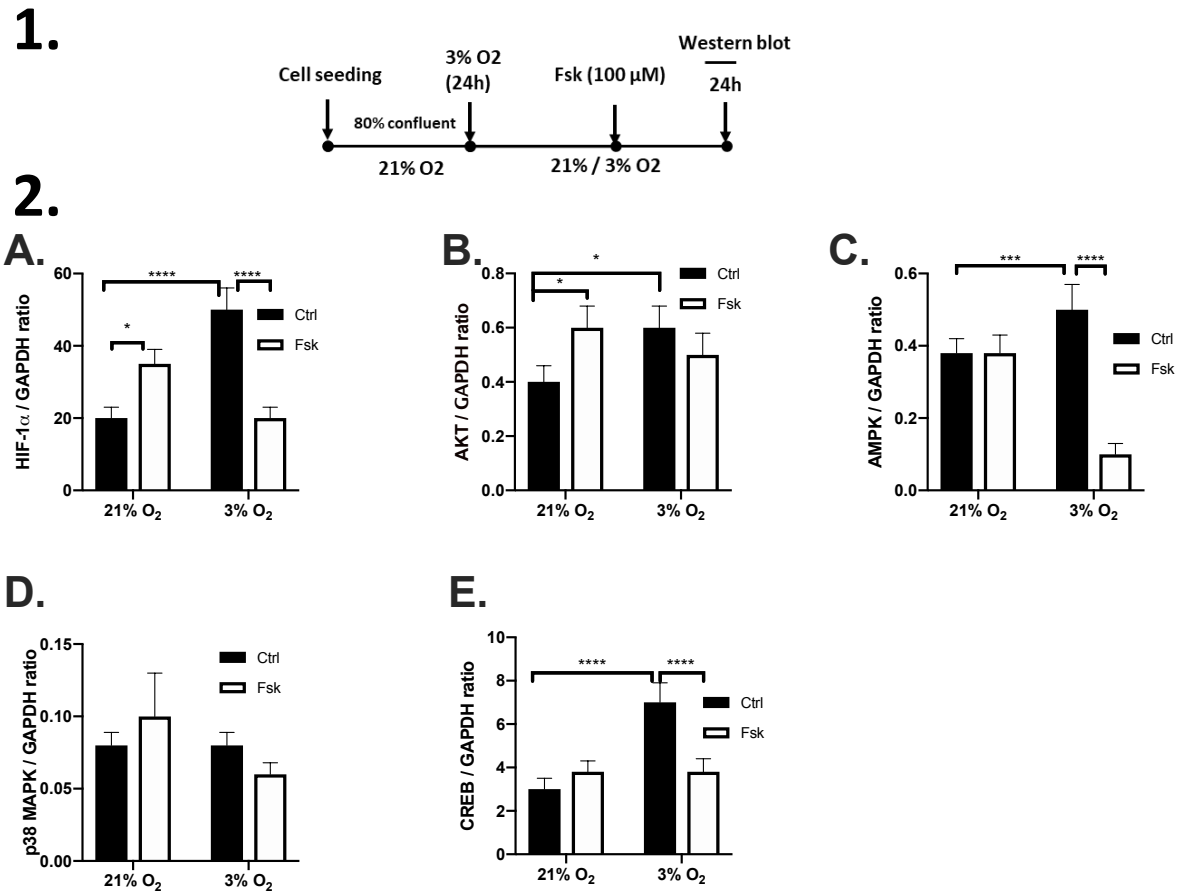


Figure 31

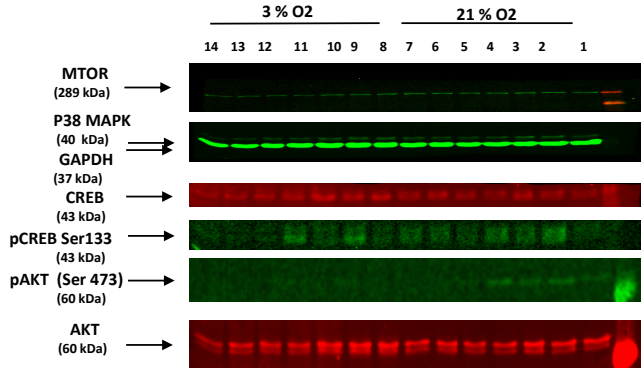
*Hypoxia increased the protein expression of HIF-1a, AKT, AMPK and CREB and reversed effects of CAMP signalling pathway on HIF-1a, reduced its effects on AMPK and CREB in BEWO cells. 1. Experimental protocol; 2. Western blot analysis showed that under low oxygen tension, increased protein expression of HIF-1a, AKT, AMPK and CREB was seen. The actions of forskolin on HIF-1a were reversed, abolished on AKT, reduced on AMPK, CREB protein expression. GAPDH was used as a loading control in each sample. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against GAPDH. p= * 0.033; *** .0002; ****p< .0001; n=3. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

Table 17

	HIF-1a		AKT		AMPK		P38 MAPK	
	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2
Ctrl	NS	↑	NS	↑	NS	↑	NS	NS

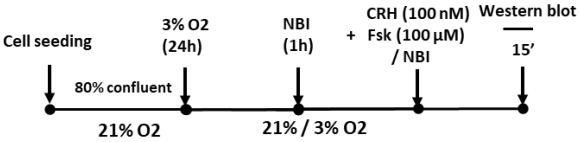
Fsk	↑	↓	↑	NS	NS	↓	NS	NS
CRH	NS	NS	NS	NS	NS	NS	NS	NS
CRH + Fsk	NS	NS	NS	NS	NS	NS	NS	NS
NBI	NS	NS	NS	NS	NS	NS	NS	NS
CRH + NBI	NS	NS	NS	NS	NS	NS	NS	NS
CRH + Fsk + NBI	NS	NS	NS	NS	NS	NS	NS	NS

Bewo 15’



1, 8 Control (Ctrl)
2, 9 Forskolin (Fsk)
3, 10 Corticotrophic releasing hormone (CRH)
4, 11 CRH + Fsk
5, 12 NBI 27914 hydrochloride
6, 13 CRH + NBI
7, 14 CRH + Fsk + NBI

1.



2.

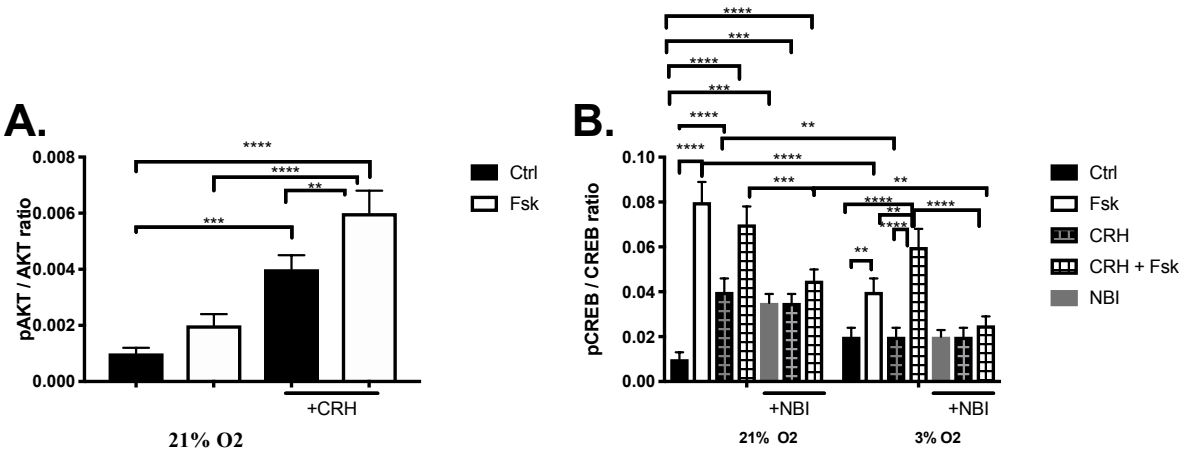


Figure 32

*forskolin increases pAKT and pCREB expression at 15'. Crh increases pcreb and hypoxia abolishes the effect of forskolin and crh on pcreb in bewo unsyncytialised cells. CRH potentiates actions of forskolin on pCREB in low oxygen tension.1.Experimental protocol; 2. (A) Forskolin increases pAKT protein expression at 15' at 21% O2. CRH increases pAKT expression in unsyncytialised and syncytialised bewo cells. (B) Forskolin and CRH (via CRHR1) increases pCREB expression. CRH has no effect on pCREB expression in syncytialised bewo cells. Hypoxia diminishes the effect of forskolin and crh on pcreb expression. Hypoxia potentiates the actions of crh on pcreb expression in syncytialised bewo cells, which is further lost on blocking CRHR1. CRH is unable to increase pCREB expression on blocking crhr1 in both unsyncytialised bewo cells and syncytialised bewo cells in both 21 and 3%O2. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against the total protein. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

Table 18

	PAKT		PCREB	
	21% O2	**3% O2**	**21% O2**	**3% O2**
Ctrl	NS	NS	NS	NS
Fsk	NS	NS	↑	↑
CRH	↑	NS	↑	NS
CRH + Fsk	↑	NS	NS	↑
NBI	NS	NS	↓	NS
CRH + NBI	NS	NS	NS	NS
CRH + Fsk + NBI	NS	NS	↓	↓

Bewo 15'

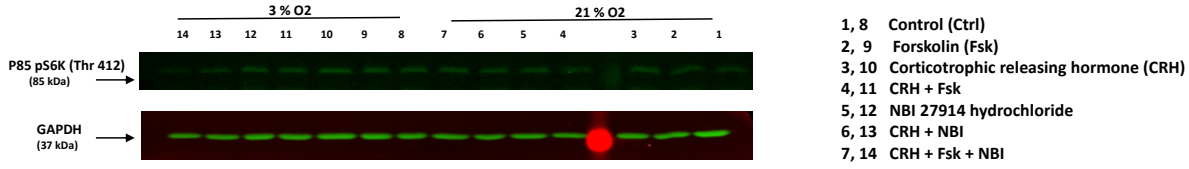


Figure 33

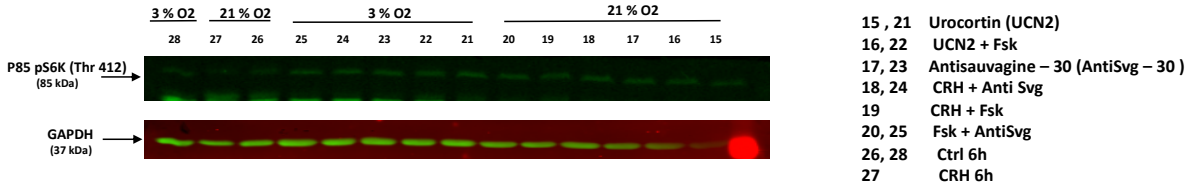
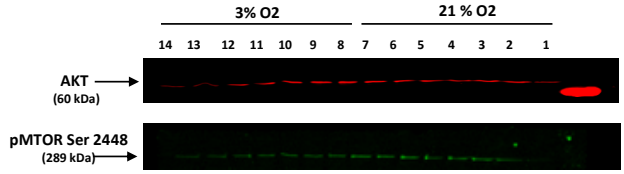


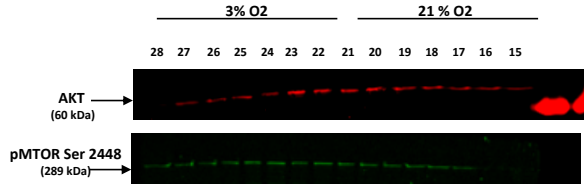
Figure 34

Bewo 1H



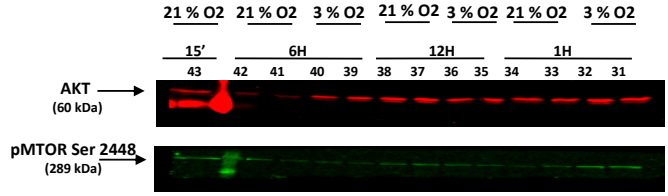
1, 8 Control (Ctrl)
2, 9 Corticotrophic releasing hormone (CRH)
3, 10 Urocortin 2 (UCN2)
4, 11 NBI 27914 hydrochloride
5, 12 CRH + NBI
6, 13 Antisauvagine – 30 (AntiSvg – 30)
7, 14 CRH + AntiSvg - 30

Figure 35



15, 22 Control (Ctrl)
16, 23 Forskolin (Fsk)
17, 24 Corticotrophic releasing hormone (CRH) + Fsk
18, 25 NBI 27914 hydrochloride + Fsk
19, 26 Antisauvagine – 30 (AntiSvg – 30) + Fsk
20, 27 Urocortin 2 (UCN2)
21, 28 UCN2 + Fsk

Figure 36



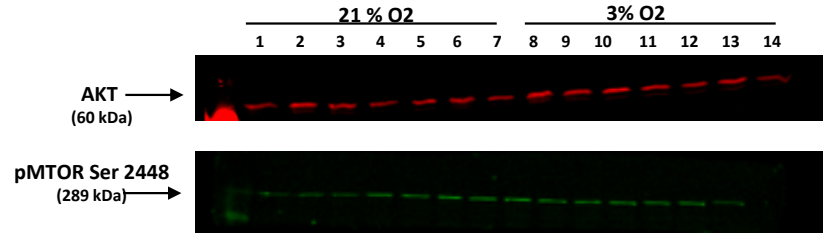
29, 31 Urocortin 2 (UCN2) } 1h
30, 32 Control (Ctrl)

33, 35 CRH } 12h
36, 38 Control (Ctrl)

39, 41 CRH } 6h
40, 42 Control (Ctrl)

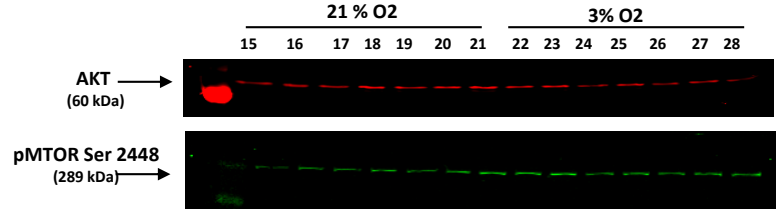
43 Forskolin 15'

Figure 37



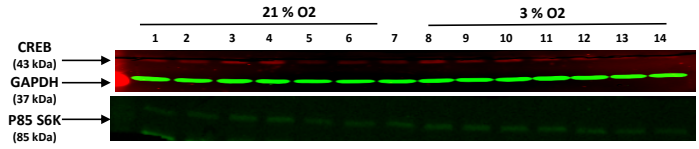
1, 8 Ctrl
2, 9 CRH
3, 10 Fsk
4, 11 NBI
5, 12 CRH + NBI
6, 13 AntiSvg
7, 14 CRH + AntiSvg

Figure 38



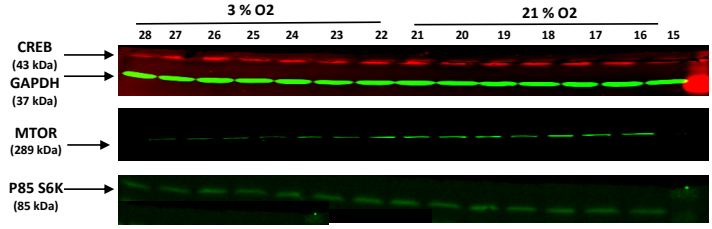
15, 22 Fsk
16, 23 CRH + Fsk
17, 24 UCN2 + Fsk
18, 25 NBI + Fsk
19, 26 AntiSvg – 30 + Fsk
20, 27 CRH + NBI + Fsk
21, 28 CRH + AntiSvg + Fsk

Figure 39



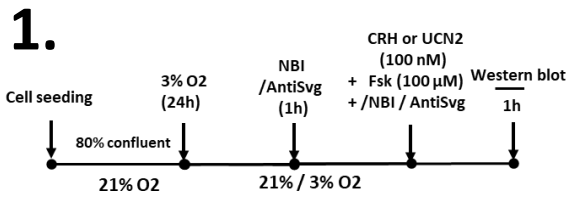
1, 8 Control (Ctrl)
2, 9 Forskolin (Fsk)
3, 10 Corticotrophic releasing hormone (CRH)
4, 11 CRH + Fsk
5, 12 NBI 27914 hydrochloride
6, 13 CRH + NBI
7, 14 CRH + Fsk + NBI

Figure 40



15 , 21 Urocortin (UCN2)
16, 22 UCN2 + Fsk
17, 23 Antisauvagine – 30 (AntiSvg – 30)
18, 24 CRH + Anti Svg
19, 25 Fsk + NBI
20, 26 Fsk + AntiSvg
21, 27 CRH + Fsk + AntiSvg

Figure 41



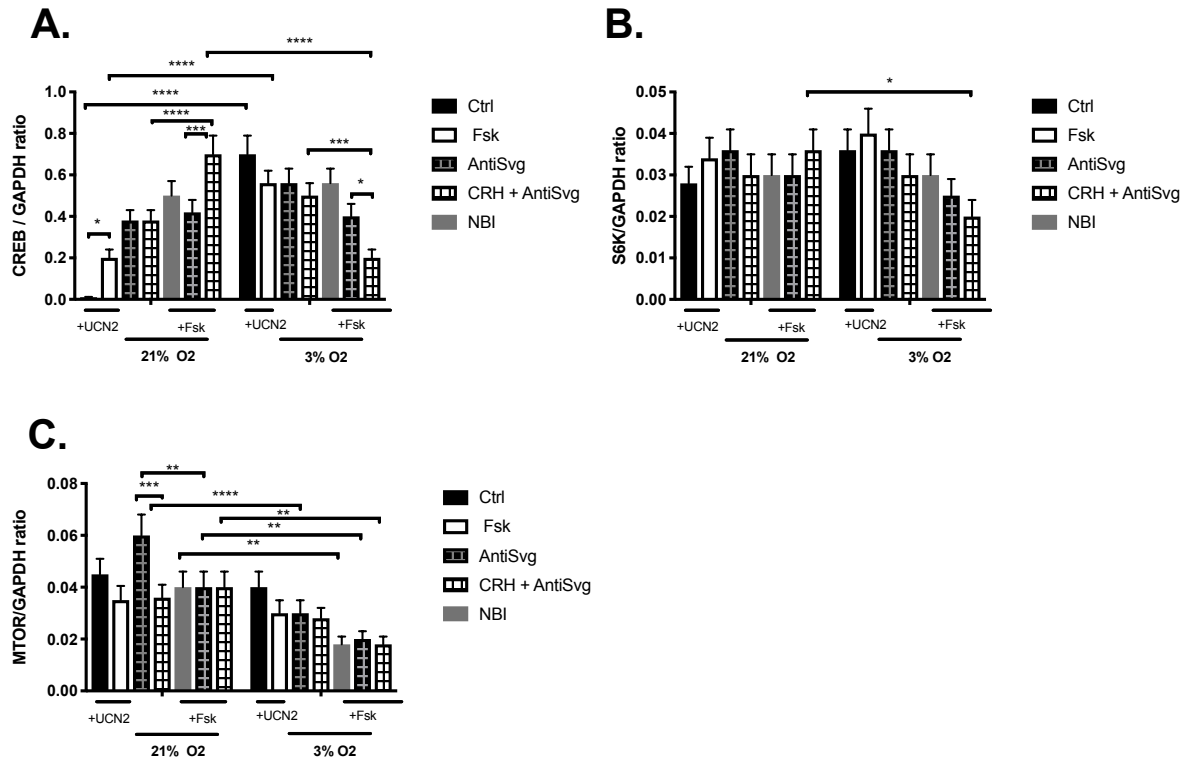


Figure 42

Table 19

1H	CREB		S6K		MTOR	
	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2
UCN2	NS	↑	NS	NS	NS	NS
UCN2 + Fsk	↑	NS	NS	NS	NS	NS
AntiSvg	↑	NS	NS	NS	NS	NS
CRH + AntiSvg	NS	NS	NS	NS	↓	NS
NBI + Fsk	NS	NS	NS	NS		NS
AntiSvg + Fsk	NS	NS	NS	NS	↓	NS
CRH + AntiSvg + Fsk	↑	↓	NS	NS	NS	NS

Bewo 24h

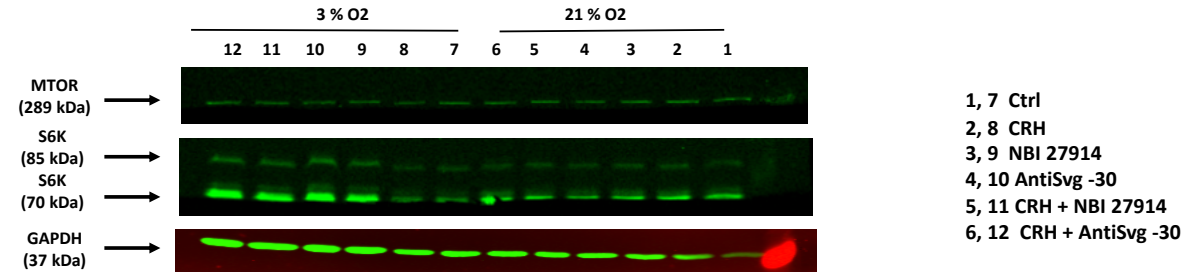


Figure 43

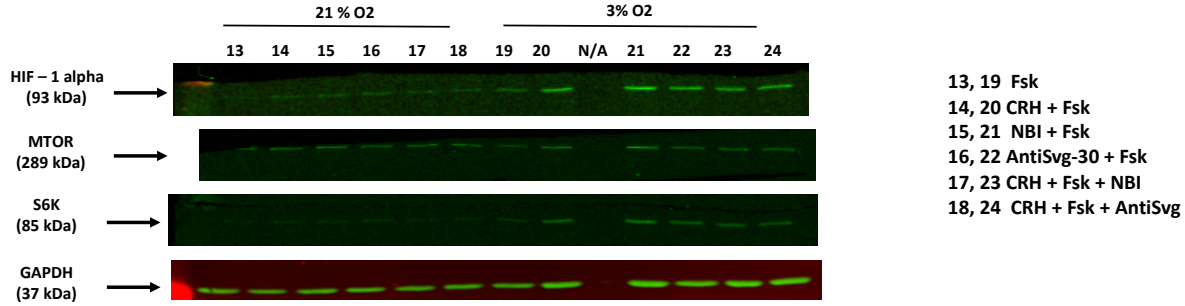


Figure 44

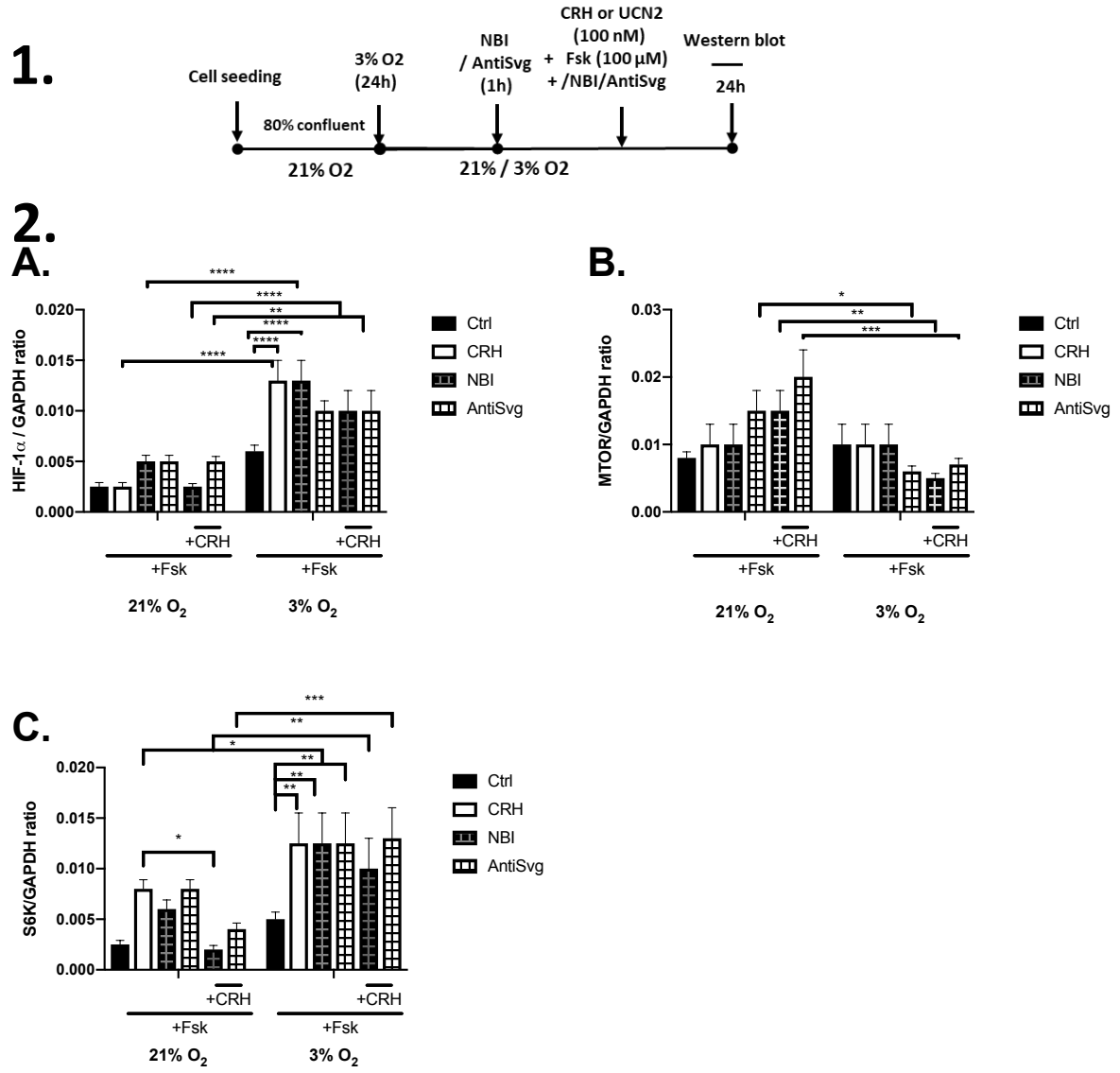


Figure 45

*Under low oxygen tension, forskolin increases HIF-1a and S6K expression. CRH increases HIF-1a in BEWO syncytialised cells. 1. Experimental protocol; 2. (A) Under low oxygen tension, forskolin increases HIF-1a expression (may be via CRHR2). Blocking crhr1 increases HIF-1a expression in BEWO syncytialised cells. CRH increases HIF—1a expression in BEWO syncytialised cells and on blocking CRHR1 or CRHR2, showed no increase in HIF-1a expression. (B) CRH has no effect on MTOR expression in BEWO syncytialised cells. (C) CRH increases S6K expression in 21 and 3% O2. In 21% O2, CRH decreases S6K protein expression on blocking CRHR1. In low oxygen tension, CRH has no effect on S6K expression by blocking CRHR1 or CRHR2. In low oxygen tension, blocking CRHR1 or CRHR2 increases S6K expression in BEWO syncytialised cells. GAPDH was used as a loading control in each sample. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against the GAPDH. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

Table 20

24H	HIF-1a		S6K		MTOR	
	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2
Fsk	NS	NS	NS	NS	NS	NS
CRH + Fsk	NS	↑	↑	↑	NS	NS

Fsk + NBI	NS	↑	NS	↑	NS	NS
Fsk + AntiSvg	NS	↑	NS	↑	NS	NS
Fsk + NBI + CRH	NS	NS	↓	NS	NS	NS
CRH + AntiSvg + Fsk	NS	NS	↓	NS	↑	NS

Figure 46

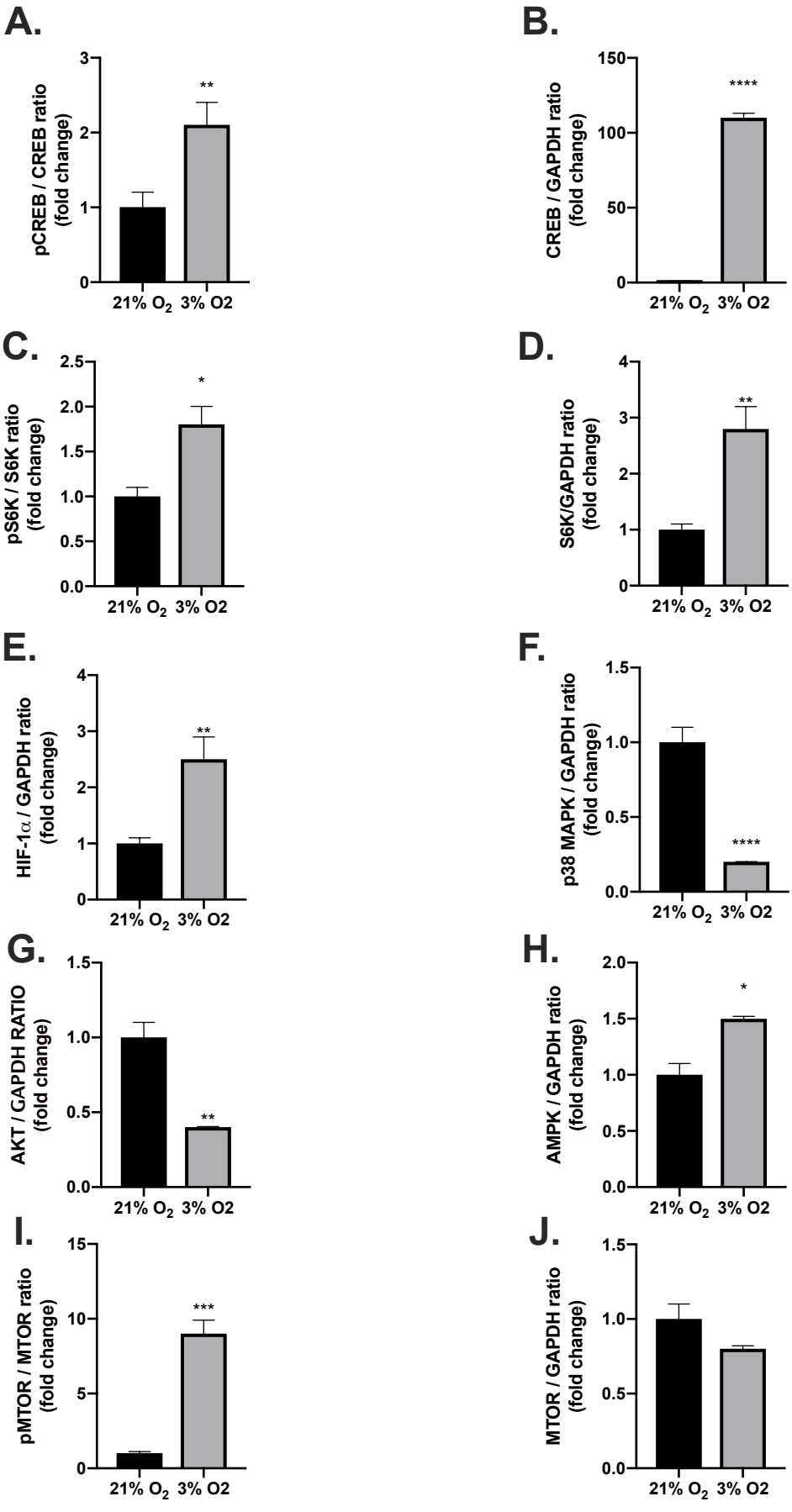
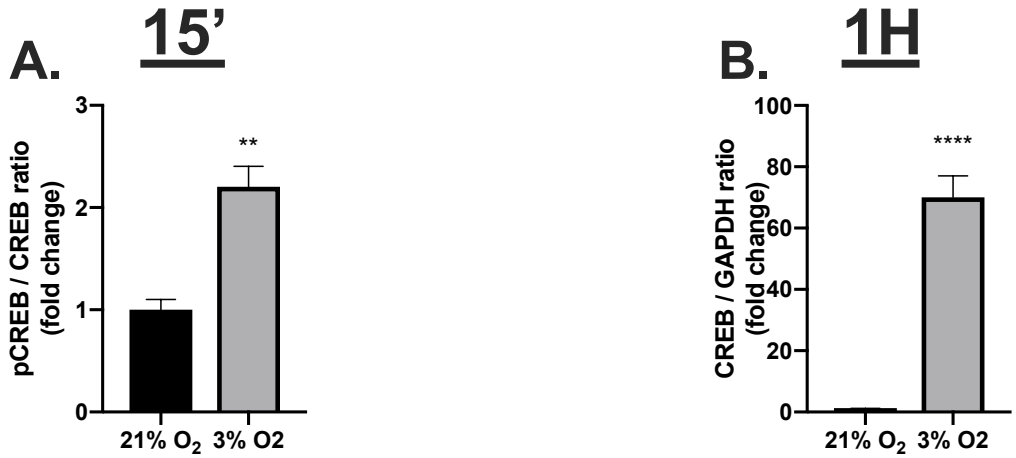

A.
pCREB / CREB ratio (fold change)
3
2
1
0
**
21% O2
3% O2
B.
CREB / GAPDH ratio (fold change)
150
100
50
0
****
21% O2
3% O2
C.
pS6K / S6K ratio (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
*
21% O2
3% O2
D.
S6K/GAPDH ratio (fold change)
4
3
2
1
0
**
21% O2
3% O2
E.
HIF-1α / GAPDH ratio (fold change)
4
3
2
1
0
**
21% O2
3% O2
F.
p38 MAPK / GAPDH ratio (fold change)
1.5
1.0
0.5
0.0
****
21% O2
3% O2
G.
AKT / GAPDH RATIO (fold change)
1.5
1.0
0.5
0.0
**
21% O2
3% O2
H.
AMPK / GAPDH ratio (fold change)
2.0
1.5
1.0
0.5
0.0
*
21% O2
3% O2
I.
pMTOR / MTOR ratio (fold change)
15
10
5
0
***
21% O2
3% O2
J.
MTOR / GAPDH ratio (fold change)
1.5
1.0
0.5
0.0
21% O2
3% O2

*Figure **47** Hypoxia increases phosphorylation of MTOR and CREB, expression of CREB, HIF-1 alpha, S6K and decreases expression of P38 MAPK, AKT in undifferentiated BEWO cells. BEWO cells were incubated with for 15 minutes for phosphorylation studies and 24h for expression studies under high (21%O2) and low oxygen tension (3% O2). In BEWO cells, under low oxygen tension (3% O2), phosphorylation of (A) CREB, (C) MTOR is increased by 50%, and 10 fold respectively. A trend towards an increase in phosphorylation of (E) S6K and expression of (J) AMPK is seen, despite failing to reach a statistical significance. Expression of (B) CREB, (G) HIF-1 alpha, (F) S6K is upregulated by 100 fold, 2 fold and 2 fold respectively under low oxygen tension. Hypoxia inhibits (H) p38 MAPK, (I) AKT by 80% and 50% respectively. No change in expression of (D) MTOR is seen. (n=3); (*p < 0.05). Data expressed as fold change after normalising phosphorylation of protein against total protein.*



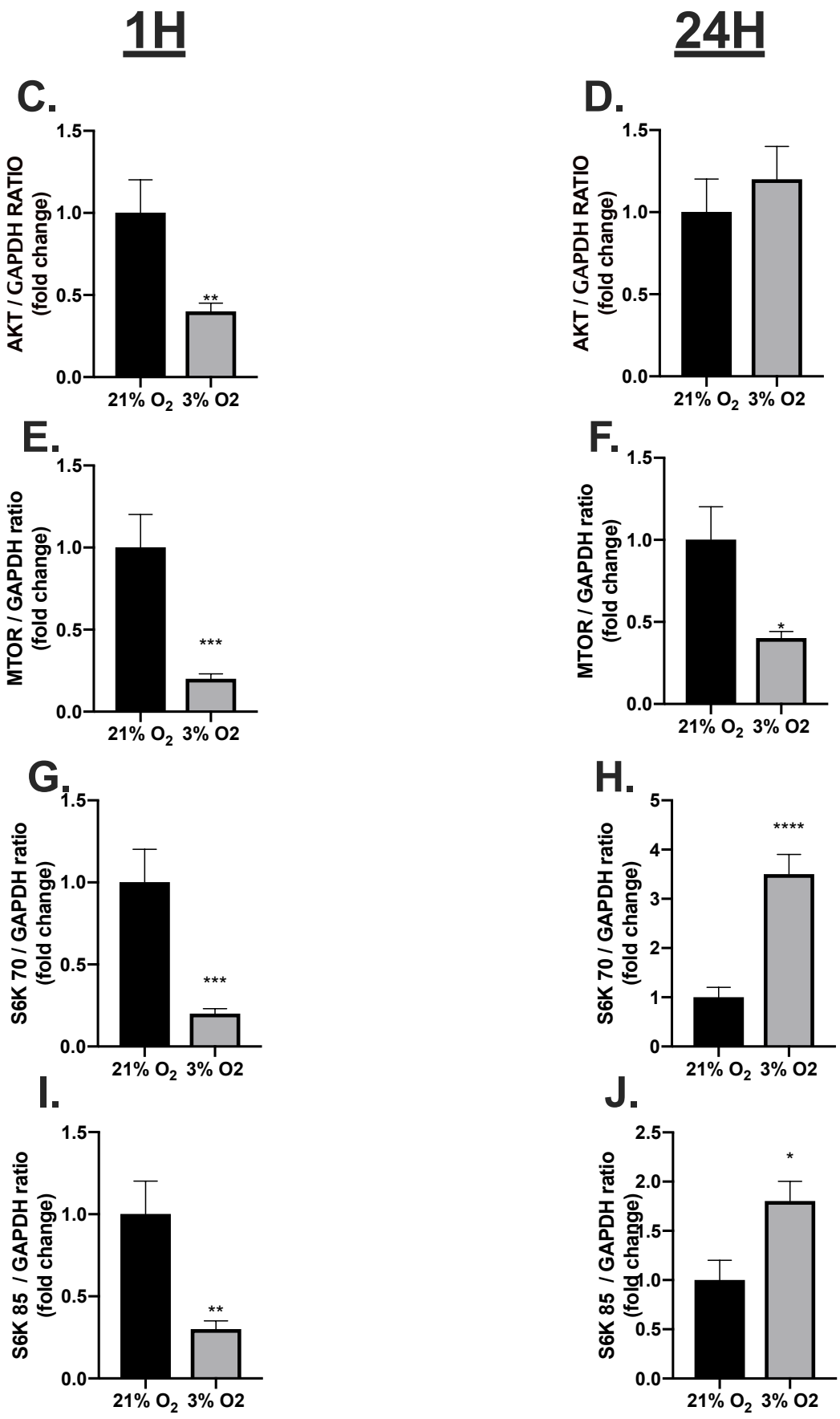

1H
24H
C.
D.
AKT / GAPDH RATIO (fold change)
1.5
1.0
0.5
0.0
**
21% O2
3% O2
E.
F.
MTOR / GAPDH ratio (fold change)
***
*
G.
H.
S6K 70 / GAPDH ratio (fold change)
5
4
3
2
1
0
****
I.
J.
S6K 85 / GAPDH ratio (fold change)
2.5
2.0
1.5

Figure 48

*At 15', hypoxia increases phosphorylation of CREB, S6K, MTOR. At 1h, hypoxia increases CREB, S6K, HIF-1a, AMPK and reduces p38 MAPK, AKT expression. No effect on MTOR expression at 1h is seen . At 1h, hypoxia reduces protein expression of AKT, MTOR, S6K70 and S6K85. At 24h, hypoxia reduces MTOR expression, increases S6K70 and S6K85 expression but no effect on AKT expression. GAPDH was used as a loading control in each sample. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against the total protein. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two tailed unpaired t test.*

Table 21

	15'	**1H**	**24H**
	3% O2	**3% O2**	**3% O2**
PCREB	↑	NS	NS
CREB	NS	↑	NS
PS6K	↑	NS	NS
S6K	NS	↑	NS
HIF-1a	NS	↑	NS
P38 MAPK	NS	↓	NS
AKT	NS	↓	NS
AMPK	NS	↑	NS
PMTOR	↑	NS	NS
MTOR	NS	↓	↓
S6K70	NS	↓	↑
S6K 85	NS	↓	↑

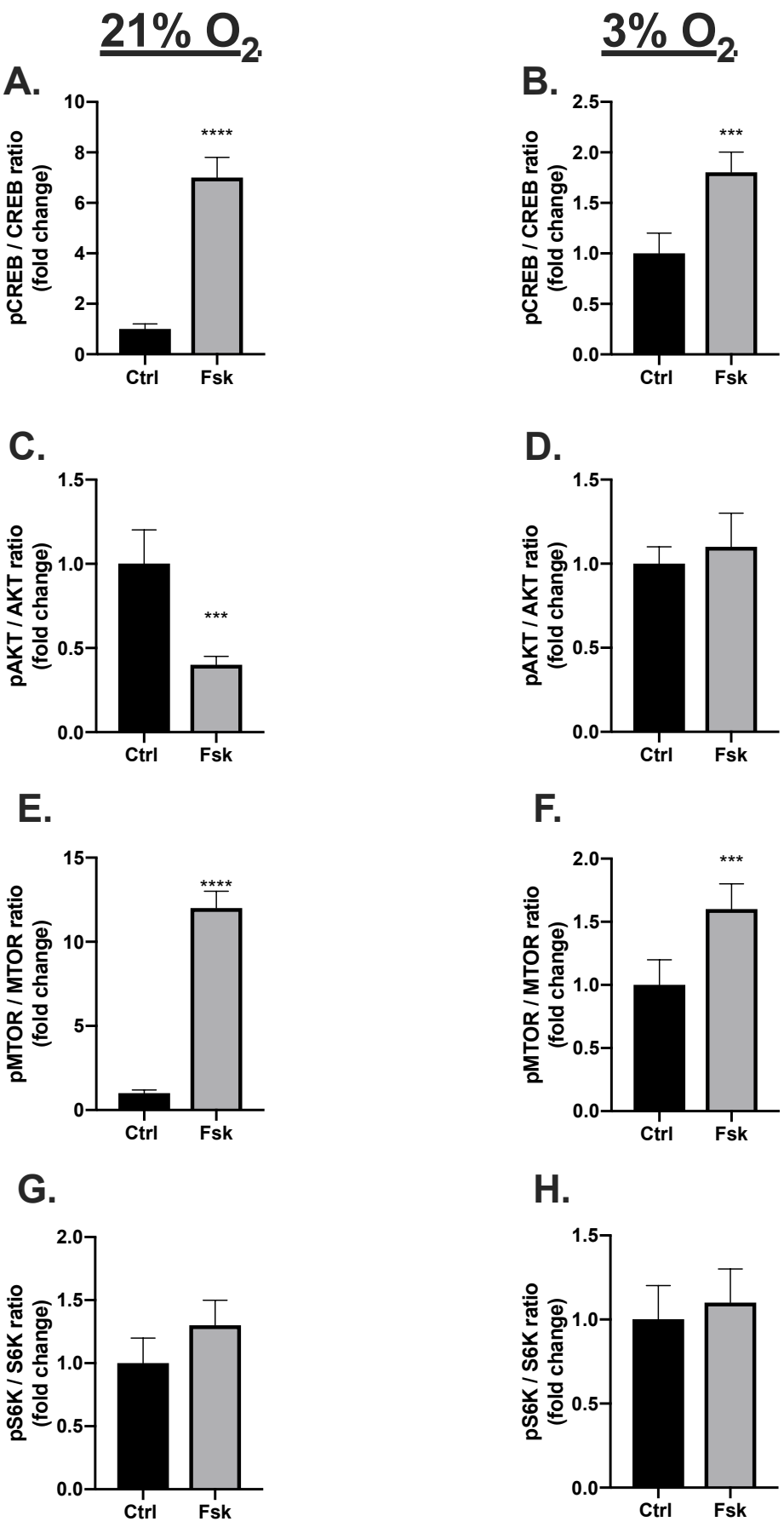

21% O2
3% O2
A.
B.
C.
D.
E.
F.
G.
H.
pCREB / CREB ratio (fold change)
pAKT / AKT ratio (fold change)
pMTOR / MTOR ratio (fold change)
pS6K / S6K ratio (fold change)
Ctrl
Fsk
****
***

Figure 49

*At 21% O2, forskolin increases phosphorylation of CREB, MTOR and reduces AKT, low oxygen tension diminishes the effect of CAMP signalling pathway on phosphorylation of CREB and MTOR and abolishes the effect on pAKT. Forskolin has no effect on pS6K at 21 and 3% O2. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against the total protein. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two tailed unpaired t test.*

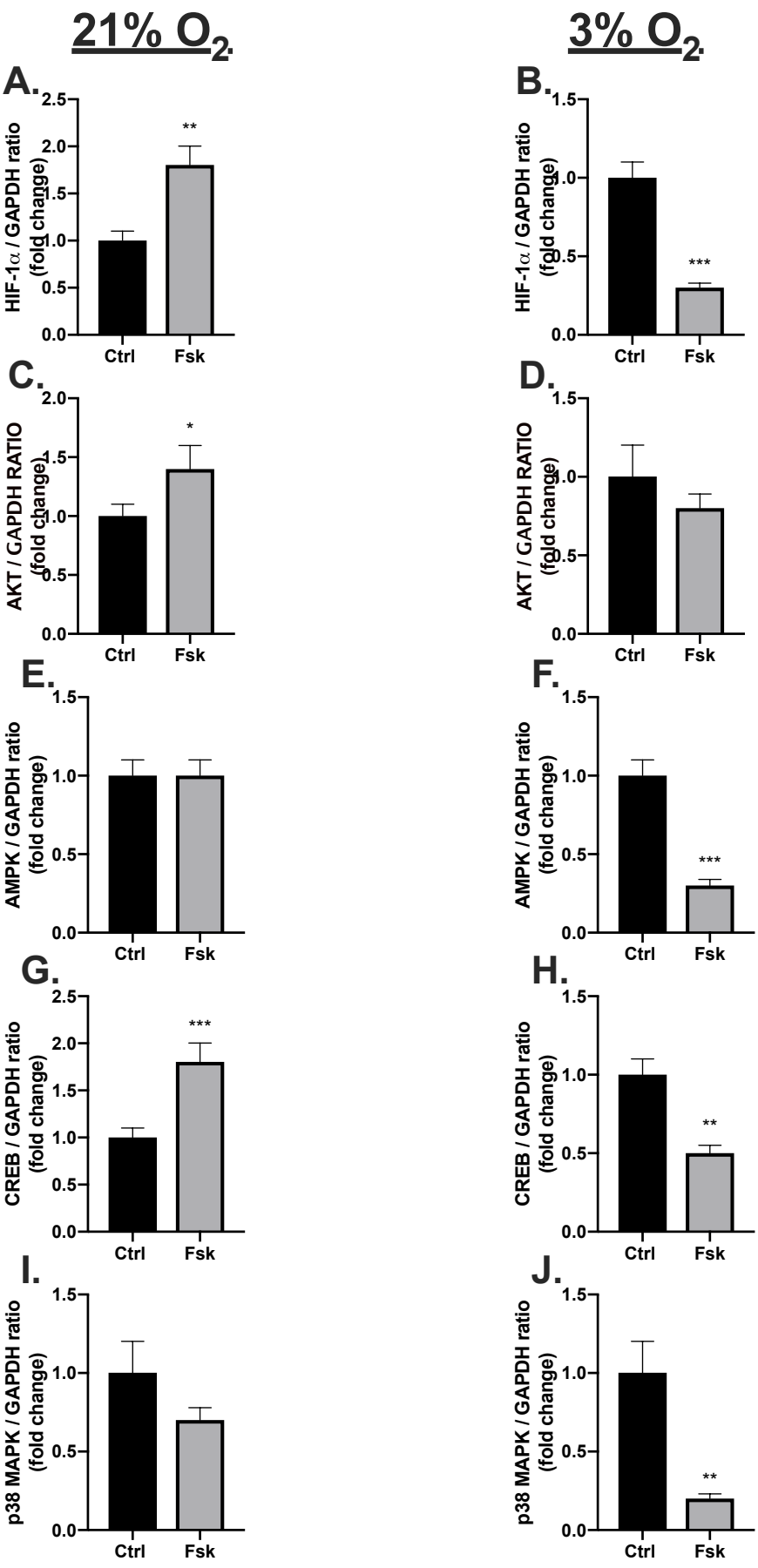

21% O2
3% O2
A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
HIF-1α / GAPDH ratio (fold change)
AKT / GAPDH RATIO (fold change)
AMPK / GAPDH ratio (fold change)
CREB / GAPDH ratio (fold change)
p38 MAPK / GAPDH ratio (fold change)
Ctrl
Fsk
*
**
***

Figure 50

*Low oxygen tension reversed the effects of camp signalling pathway on HIF-1a and CREB expression and blocks the effect on AKT expression and further reduces AMPK and p38 expression. GAPDH was used as a loading control in each sample. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against the total protein. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two tailed unpaired t test.*

Table 22

Fsk	**21%O2**	**3%O2**
PCREB	↑	↑
PAKT	↓	NS
PMTOR	↑	↑
PS6K	NS	NS
HIF-1a	↑	↓
AKT	↑	NS
AMPK	NS	↓
CREB	↑	↓
P38 MAPK	NS	↓

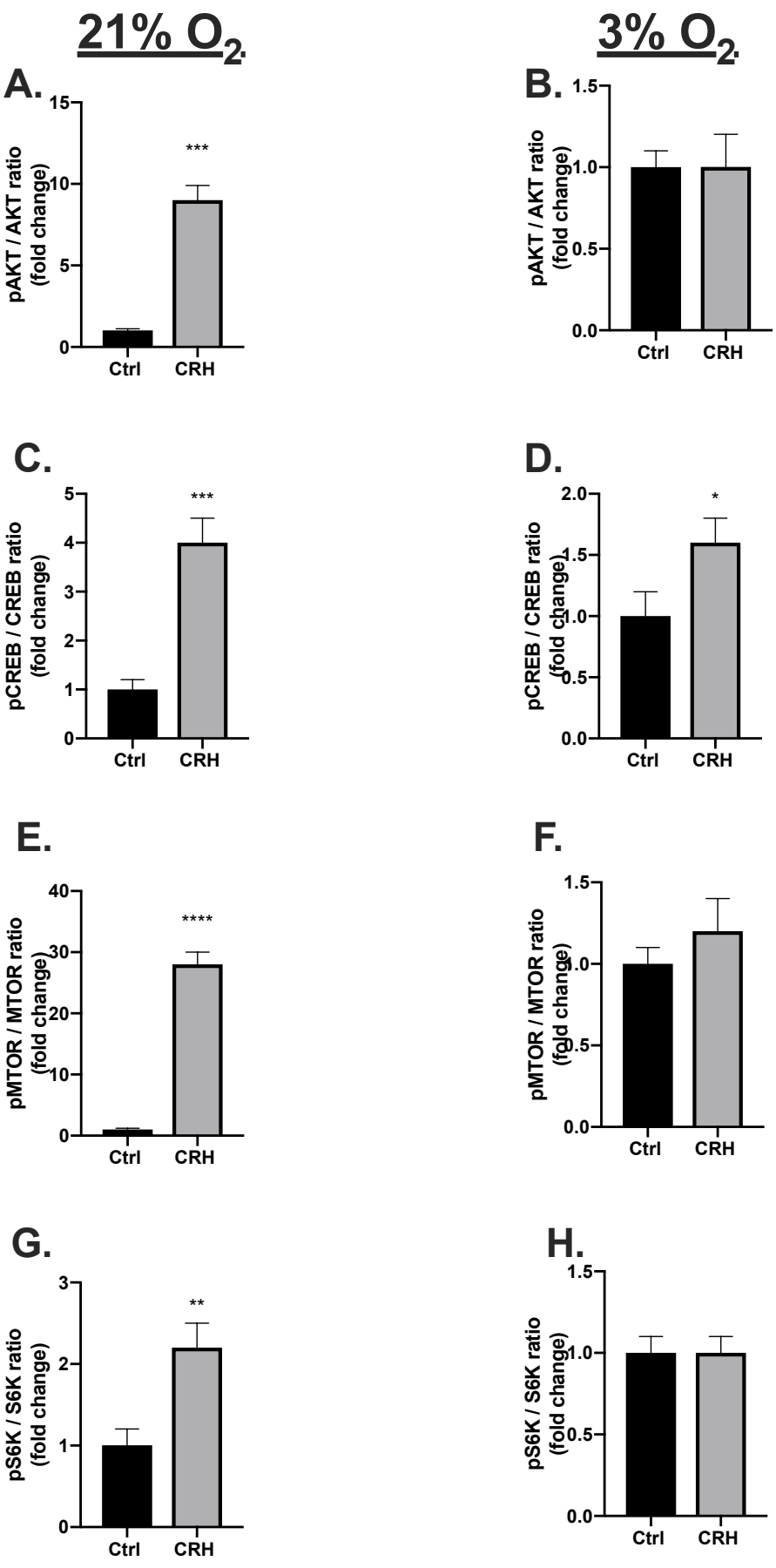

21% O2
3% O2
A.
B.
C.
D.
E.
F.
G.
H.
pAKT / AKT ratio (fold change)
pCREB / CREB ratio (fold change)
pMTOR / MTOR ratio (fold change)
pS6K / S6K ratio (fold change)
Ctrl
CRH
***
*
****
**

Figure 51

*At 21% O2, CRH increases phosphorylation of AKT, CREB, MTOR and S6K. Hypoxia abolishes the effect on AKT, MTOR, S6K but diminishes effect on CREB. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against the total protein. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two tailed unpaired t test.*

Table 23

CRH	21%O2	3%O2
PAKT	↑	NS
PCREB	↑	↑
PMTOR	↑	NS
PS6K	↑	NS

BEWO 15’

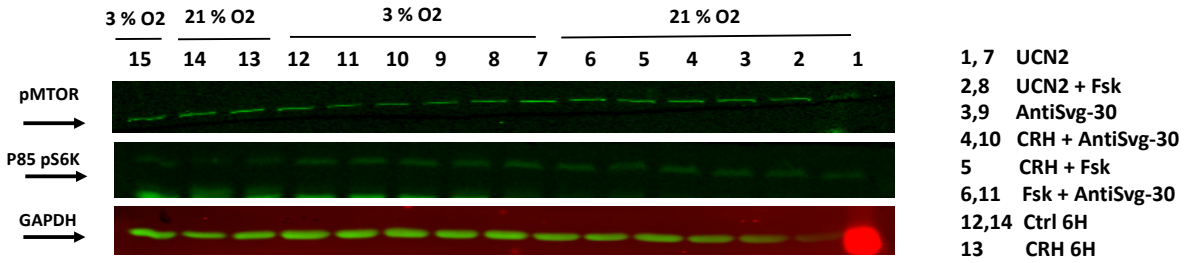
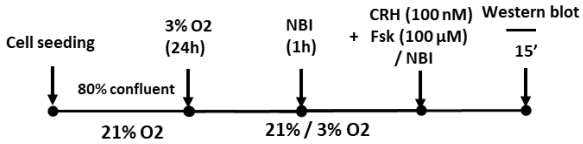


Figure 52

1.



2.

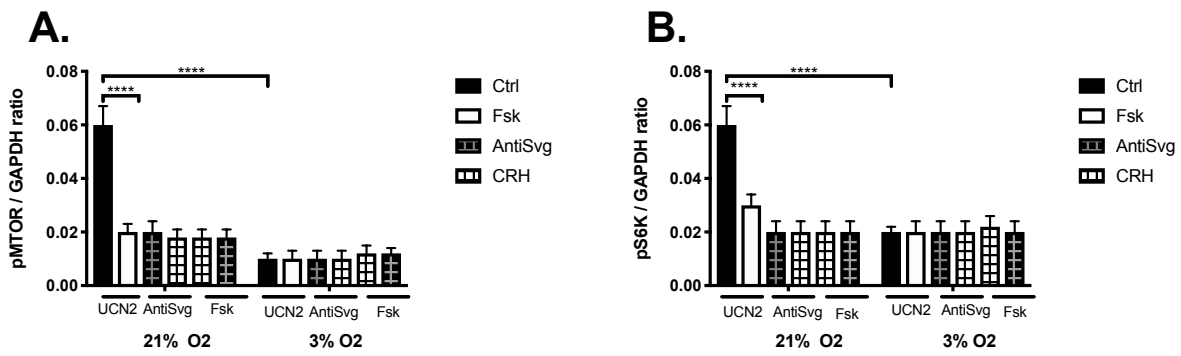


Figure 53

Table 24

15'	PMTOR		PS6K	
	21% O2	3% O2	21% O2	3% O2
UCN2	NS	↓	NS	↓
UCN2 + Fsk	↓	NS	↓	NS
AntiSvg	NS	NS	NS	NS
AntiSvg + Fsk	NS	NS	NS	NS
CRH + Fsk	NS	NS	NS	NS
Fsk + AntiSvg	NS	NS	NS	NS

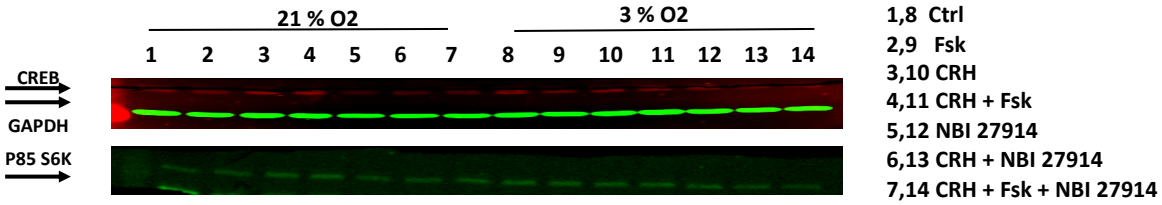


Figure 54

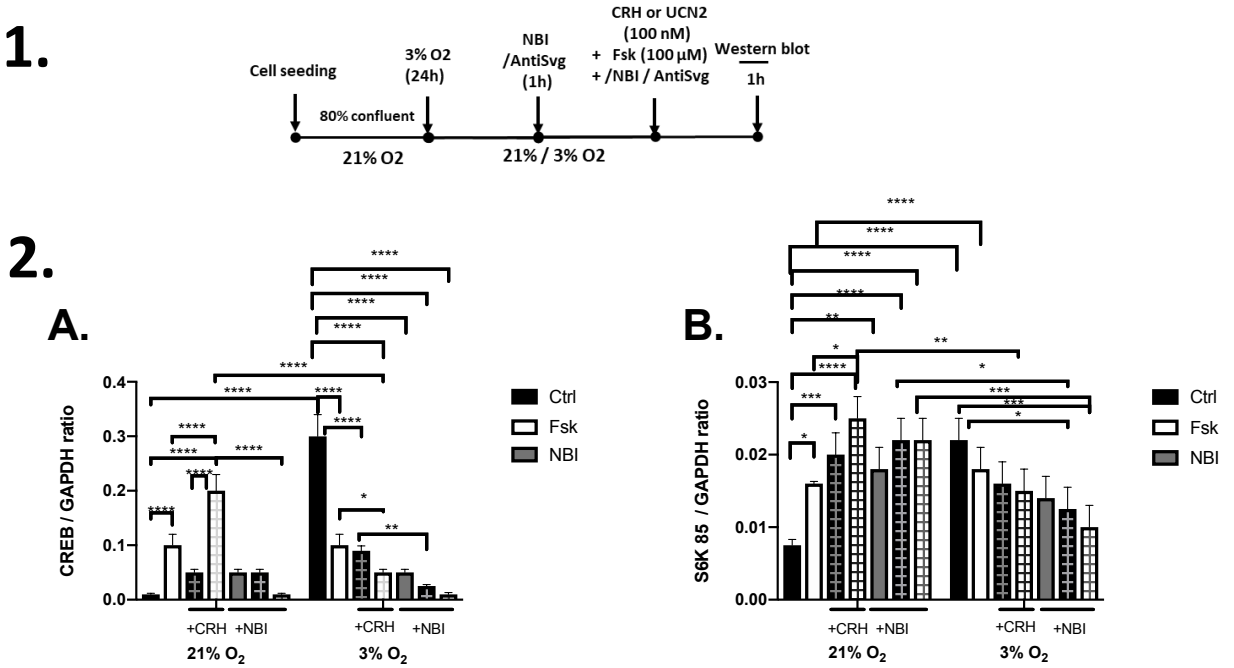


Figure 55

*At 1h at 21% O2, forskolin increases CREB expression. CRH increases CREB expression in BEWO syncytialised cells, which is reduced on blocking CRHR1. CRH has no effect on CREB expression and blocking CRHR1 has no further effect. At 3% O2, hypoxia increases CREB expression. Low oxygen tension reverses the effect of forskolin and CRH on CREB expression and blocks the effect of CRH in BEWO syncytialised cells. Blocking CRHR1 diminishes the effect of CRH on CREB expression. At1h, at 21% O2, forskolin and CRH increases S6K85 expression. CRH potentiates CREB expression in BEWO syncytialised cells. CRH does not increase S6K85 expression in unsyncytialised and syncytialised BEWO cells on blocking CRHR1. At 3% O2, hypoxia increases S6K85 expression. Hypoxia blocks the effect of forskolin and CRH on S6K85 expression. Hypoxia blocks the effect of CRH on s6k85 expression in BEWO syncytialised cells. CRH has no further effect on S6K85 expression in unsyncytialised and syncytialised BEWO cells on blocking CRHR1. GAPDH was used as a loading control in each sample. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against GAPDH. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

Table 25

	CREB		**S6K85**	
	21% O2	3% O2	21% O2	3% O2
Ctrl	NS	↑	NS	↑
Fsk	↑	↑	↑	NS
CRH	NS	↑	↑	NS
CRH + Fsk	↑	↑	↑	NS
NBI	NS	↑	↑	NS
CRH + NBI	NS	NS	NS	NS
CRH + Fsk + NBI	↓	↓	NS	NS

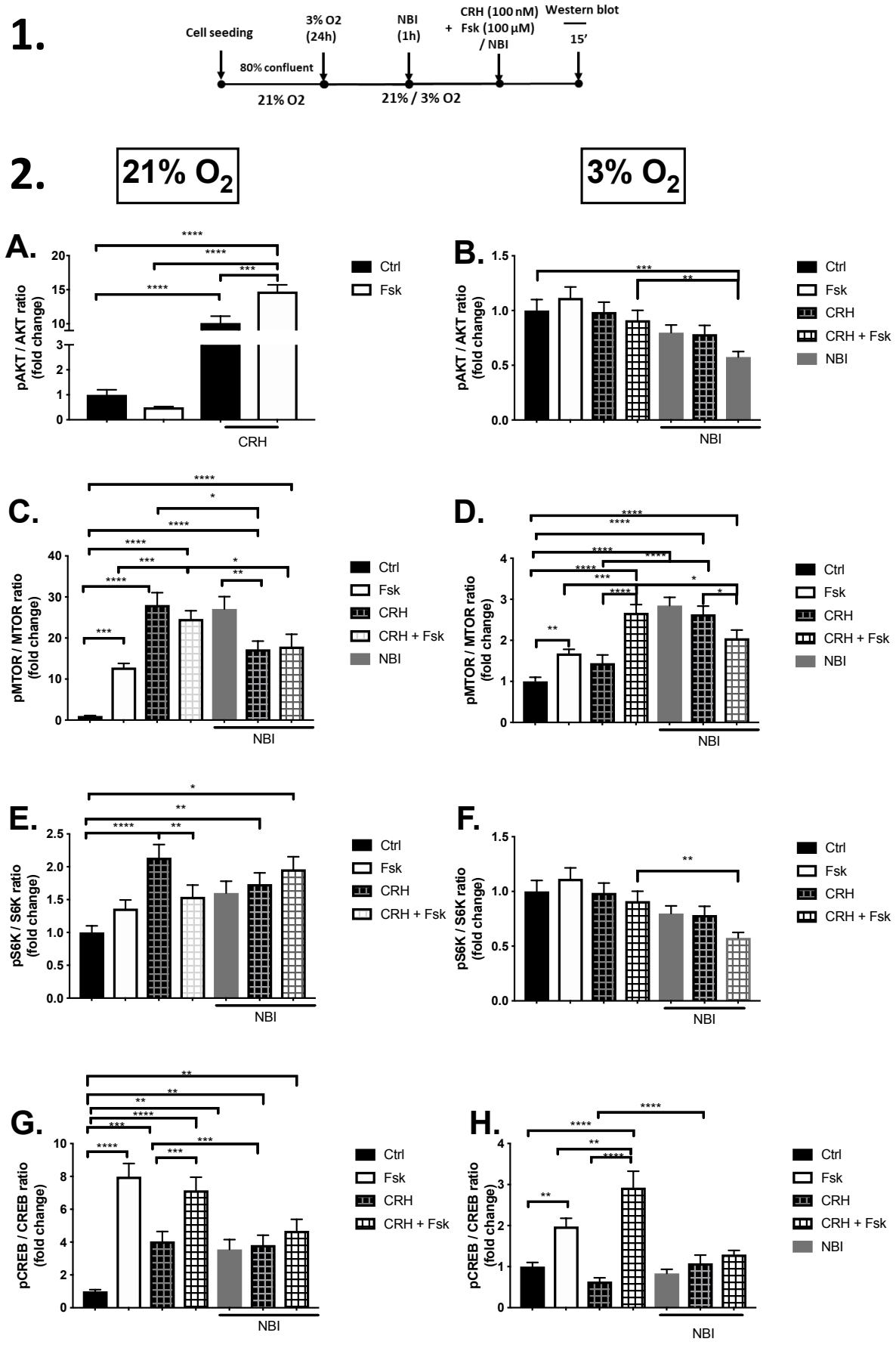

1.
Cell seeding
3% O2 (24h)
NBI (1h)
CRH (100 nM) + Fsk (100 µM) / NBI
Western blot
15'
80% confluent
21% O2
21% / 3% O2
2.
21% O2
3% O2
A.
B.
C.
D.
E.
F.
G.
H.
pAKT / AKT ratio (fold change)
pMTOR / MTOR ratio (fold change)
pS6K / S6K ratio (fold change)
pCREB / CREB ratio (fold change)
Ctrl
Fsk
CRH
CRH + Fsk
NBI

Figure 56

At 21% O2, forskolin decreases phosphorylation of AKT. CRH increases phosphorylation of AKT in unsyncytialised and syncytialised BEWO cells. Hypoxia abolishes all the effects. Blocking CRHR1 further makes no difference.

At 21% O2, forskolin and CRH increases phosphorylation of MTOR. CRH further increases phosphorylation of MTOR in BEWO syncytialised cells. CRH reduces phosphorylation of MTOR by blocking CRHR1 in BEWO unsyncytialised and syncytialised cells. Hypoxia diminishes the effects of forskolin and CRH on pMTOR in unsyncytialised and syncytialised BEWO cells. Crh has no effect on pmtor on Blocking CRHR1. On blocking crhr1, crh decreases pmtor in bewo syncytialised cells.

Crh increases ps6k in unsyncytialised but not in syncytialised bewo cells. Forskolin has no effect on ps6k in bewo cells. Crh has no effect on ps6k in unsyncytialised and syncytialised bewo cells on Blocking crhr1. Hypoxia blocks effect of crh on ps6k in bewo cells. Crh reduces ps6k in bewo syncytialised cells on blocking CRHR1.

Forskolin and crh increases pcreb. Crh has no effect on pcreb in syncytialised bewo cells. Crh has no effect on pcreb in unsyncytialised and syncytialised bewo cells on blocking crhr1. Hypoxia diminishes forskolin effect on pcreb. Crh has no effect on pcreb. Crh has increased effect on pcreb in bewo syncytialised cells. Crh decreses pcreb in bewo syncytialised cells on blocking crhr1. Crh has no effect on pcreb in bewo cells on blocking crhr1.

*GAPDH was used as a loading control in each sample. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against total protein. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

Table 26

15'	PAKT		PMTOR		PS6K		PCREB	
	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2
Fsk	NS	NS	↑	↑	NS	NS	↑	↑
CRH	↑	NS	↑	NS	↑	NS	↑	NS
CRH + Fsk	↑	NS	↑	↑	NS	NS	NS	↑
NBI	NS	NS	↑	↑	NS	NS	↑	NS
CRH + NBI	NS	NS	↓	↑	NS	NS	NS	NS
CRH + Fsk + NBI	NS	↓	↓	↓	NS	↓	↓	↓

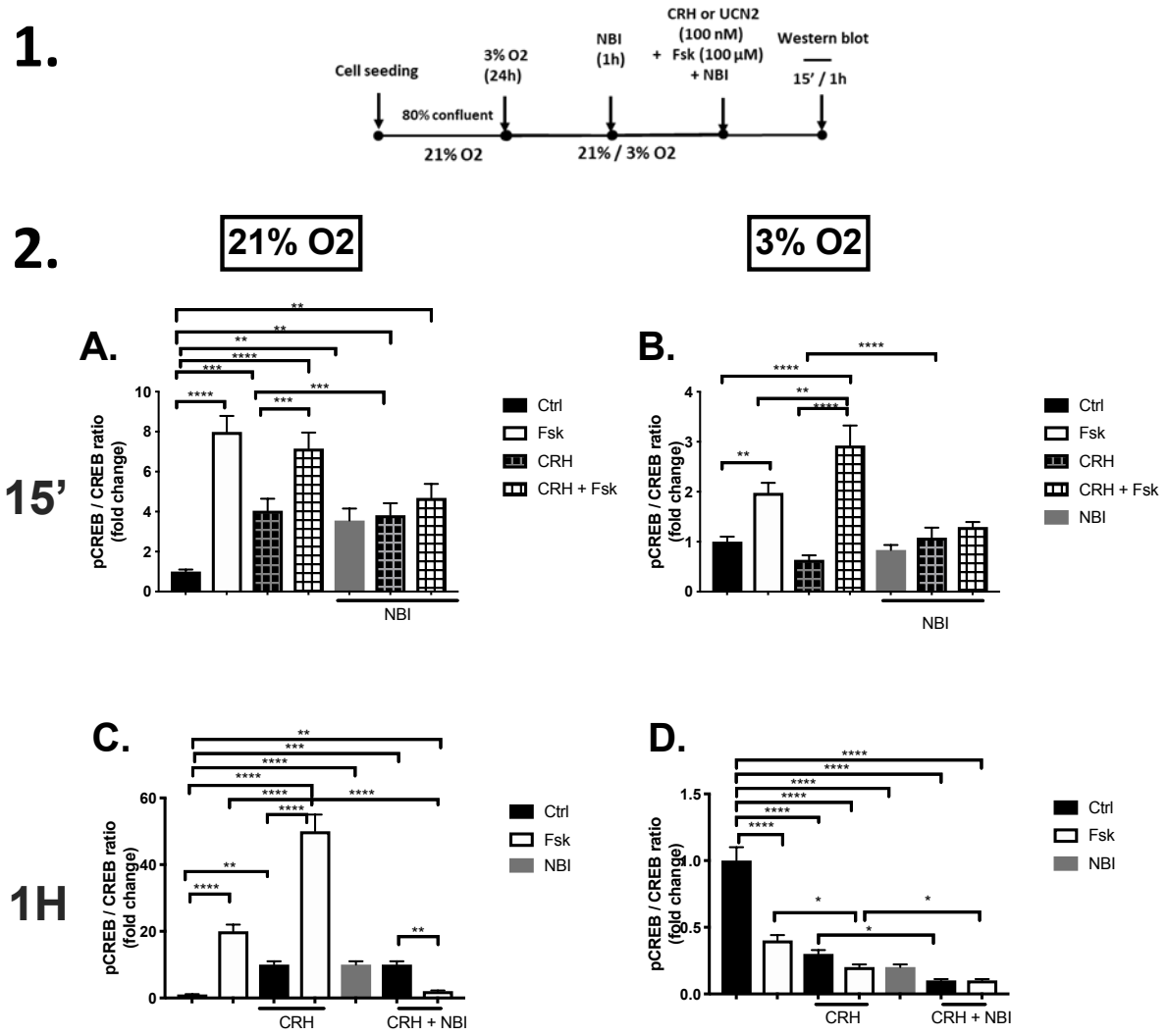


Figure 57

Time dependent increase in pcreb is seen with crh and forskolin at 21% O2. At 15', Forskolin and crh increases pcreb. Crh has no effect on pcreb in syncytialised bewo cells. Crh has no effect on pcreb in unsyncytialised and syncytialised bewo cells on blocking crhr1. Hypoxia diminishes forskolin effect on pcreb. Crh has no effect on pcreb. Crh has increased effect on pcreb in bewo syncytialised cells. Crh decreses pcreb in bewo syncytialised cells on blocking crhr1. Crh has no effect on pcreb in bewo cells on blocking crhr1. At 1h, forskolin and crh increases pcreb. Crh increases pcreb in syncytialised bewo cells. Crh is unable to increase pcreb after blocking crhr1. Crh reduces pcreb in syncytialised bewo cells on blocking crhr1.

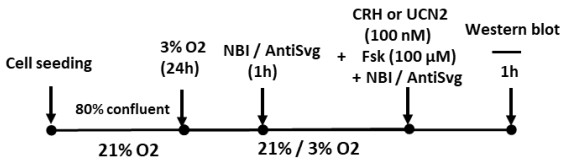
*Hypoxia reverses actions of forskolin and crh on pcreb. Hypoxia reverses the actions of crh on pcreb in syncytialised bewo cells. Crh reduces pcreb in unsyncytialised and syncytialised bewo cells on blocking crhr1. GAPDH was used as a loading control in each sample. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against total protein. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

Table 27

PCREB	**15'**		**1H**	
	21% O2	**3% O2**	**21% O2**	**3% O2**
Ctrl	NS	NS	NS	NS
Fsk	↑	↑	↑	↓
CRH	↑	NS	↑	↓

CRH + Fsk	NS	↑	↑	↓
NBI	↑	NS	↑	↓
CRH + NBI	NS	NS	NS	↓
CRH + Fsk + NBI	NS	↓	↓	↓

1.



2.

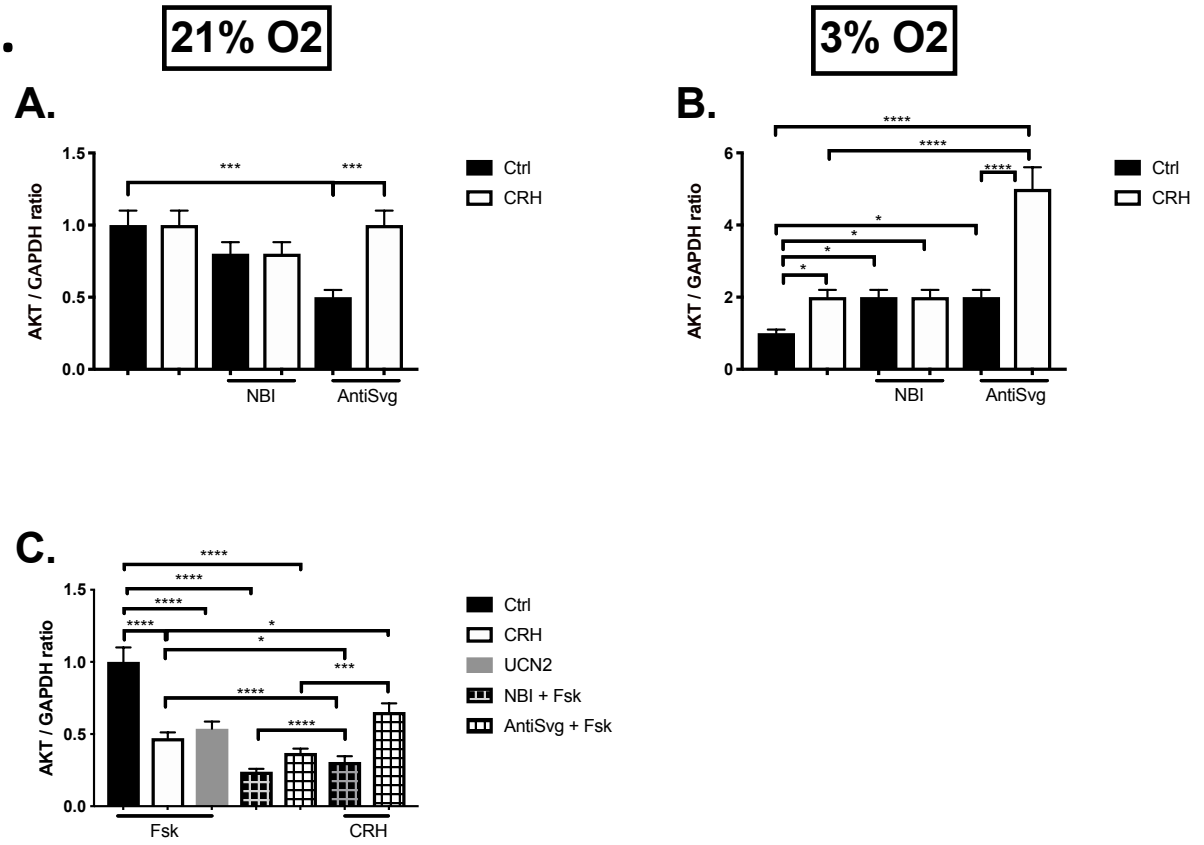


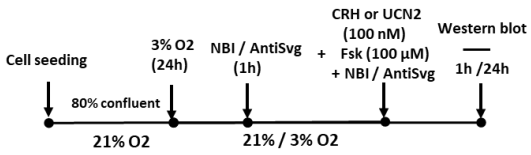
Figure 58

At 1h, Crh increases akt expression at 3% O2 via crhr1. Crh has no effect on akt expression on blocking crhr1 at 21% and 3% O2. Crh increases akt expression on blocking crhr2 at 21% o2. On blocking crhr2, at 3% o2, crh increases akt expression.

Table 28

PCREB	15'		1H	
	21% O2	3% O2	21% O2	3% O2
Ctrl	NS	NS	NS	NS
Fsk	↑	↑	↑	↓
CRH	↑	NS	↑	↓
CRH + Fsk	NS	↑	↑	↓
NBI	↑	NS	↑	↓
CRH + NBI	NS	NS	NS	↓
CRH + Fsk + NBI	NS	↓	↓	↓

1.



2.

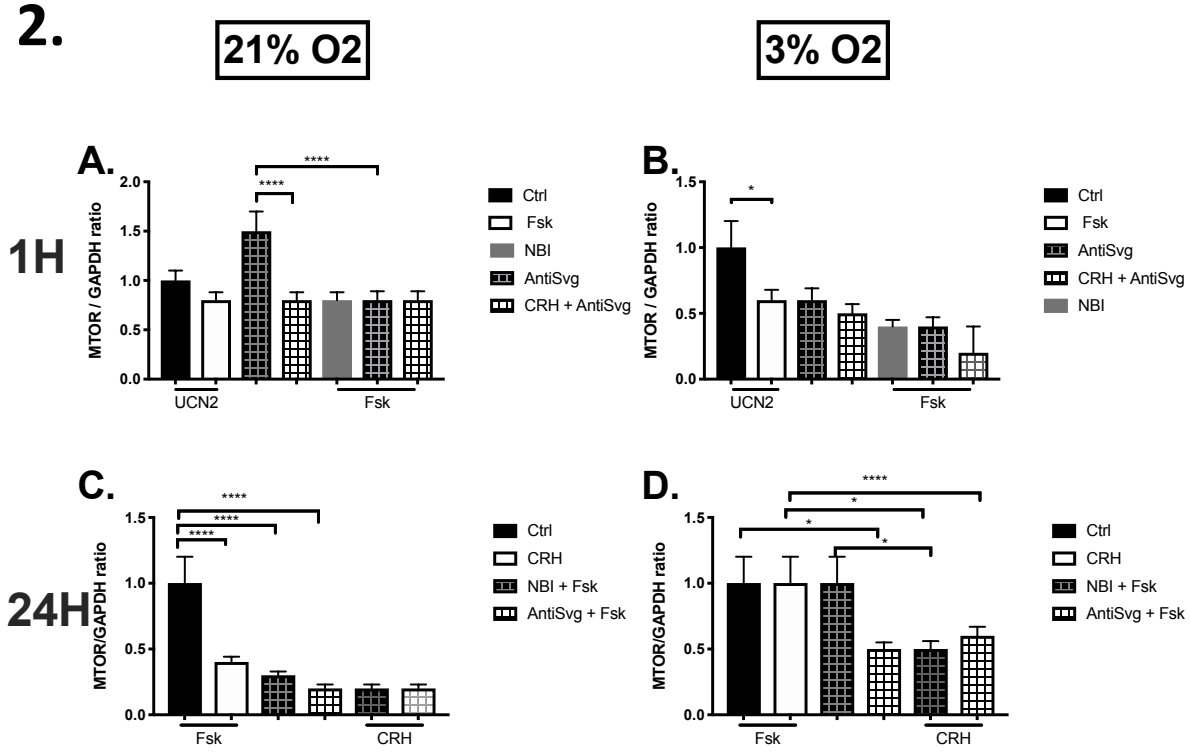


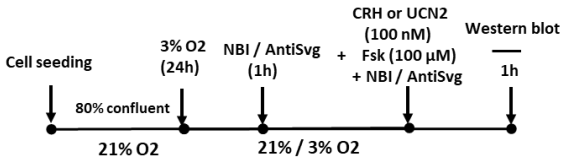
Figure 59

At 24h, at 21% o2, crh decreases mtor expression in syncytialised bewo cells. Blocking crhr1 or crhr2 reduces mtor expression in syncytialised bewo cells. Crh has no effect on mtor expression on blocking crhr1 or crhr2. At 24h, at 3% o2, crh is unable to reduce mtor expression in syncytialised bewo cells. Blocking crhr2 but not crhr1 reduces mtor expression in syncytialised bewo cells. Crh reduces mtor expression on blocking crhr1 but not crhr2 in syncytialised bewo cells.

Table 29

MTOR	**1H**		**24H**	
	21% O2	**3%O2**	**21%O2**	**3%O2**
UCN2	NS	NS	NS	NS
UCN2 + Fsk	NS	↓	NS	NS
NBI	NS	NS	NS	NS
AntiSvg	NS	NS	NS	NS
NBI + Fsk	NS	NS	NS	NS
Fsk + AntiSvg	NS	NS	NS	NS
CRH + AntiSvg	↓	NS	NS	NS
Fsk	NS	NS	NS	NS
CRH + Fsk	NS	NS	↓	NS
NBI + Fsk	NS	NS	↓	NS
AntiSvg + Fsk	NS	NS	↓	NS
CRH + NBI + Fsk	NS	NS	NS	NS
CRH + AntiSvg + Fsk	NS	NS	NS	NS

1.



1H

2.

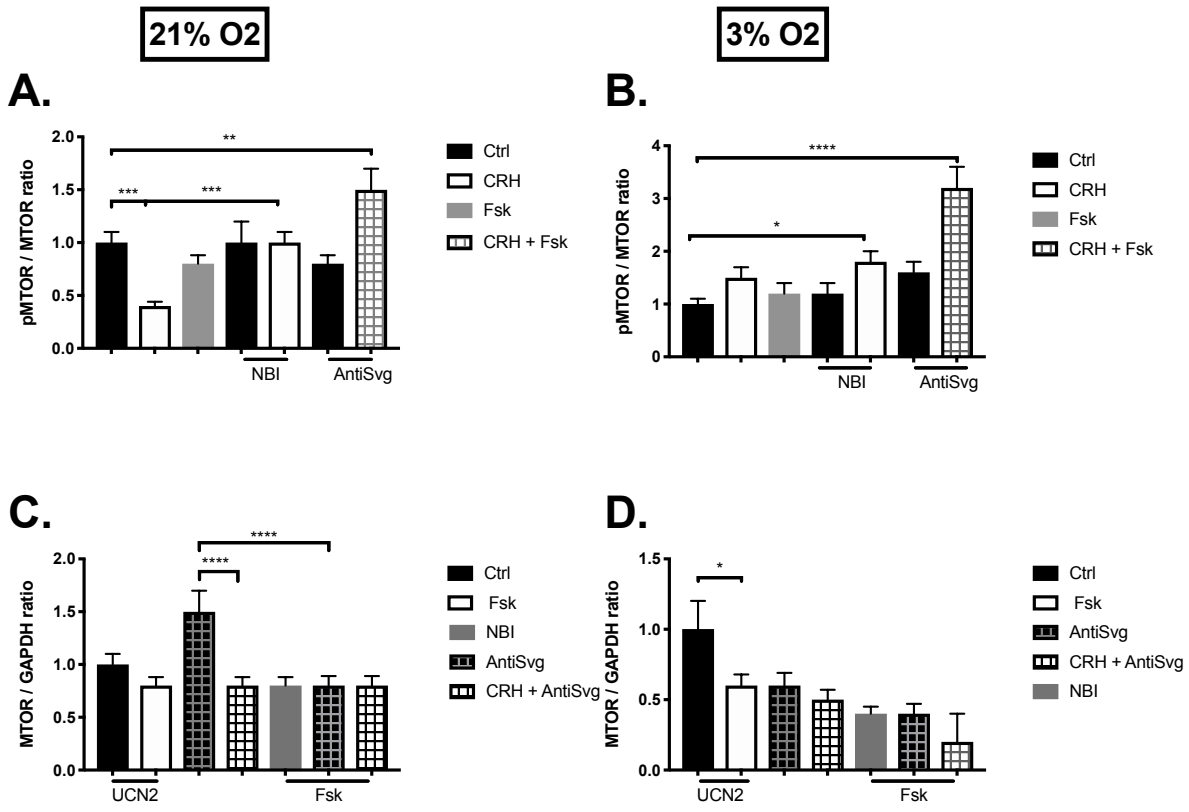


Figure 60

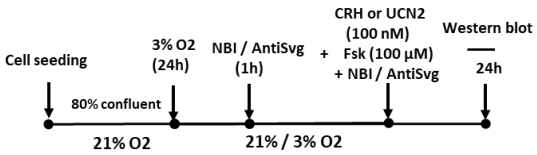
At 1h, crh decreases pmtor expression. Forskolin has no effect on pmtor expression. Crh has no effect on pmtor expression on blocking crhr1. At 3% o2, the ability to reduce pmtor by crh is lost. Crh increases pmtor on blocking crhr2 more than blocking crhr1.

Table 30

1H	PMTOR		MTOR	
	21% O2	3% O2	21% O2	3% O2
Ctrl	NS	NS	NS	NS
CRH	↓	NS	NS	NS
Fsk	NS	NS	NS	NS
CRH + Fsk	NS	NS	NS	NS

NBI	NS	NS	NS	NS
CRH + NBI	↑	NS	NS	NS
AntiSvg	NS	NS	NS	NS
CRH + AntiSvg	NS	NS	↓	NS
CRH + AntiSvg + Fsk	↑	NS	NS	NS
UCN2	NS	NS	NS	NS
UCN2 + Fsk	NS	↓	NS	↓
Fsk + NBI	NS	NS	NS	NS
Fsk + AntiSvg	NS	NS	↓	NS
CRH + Fsk + AntiSvg	NS	NS	NS	NS

1.



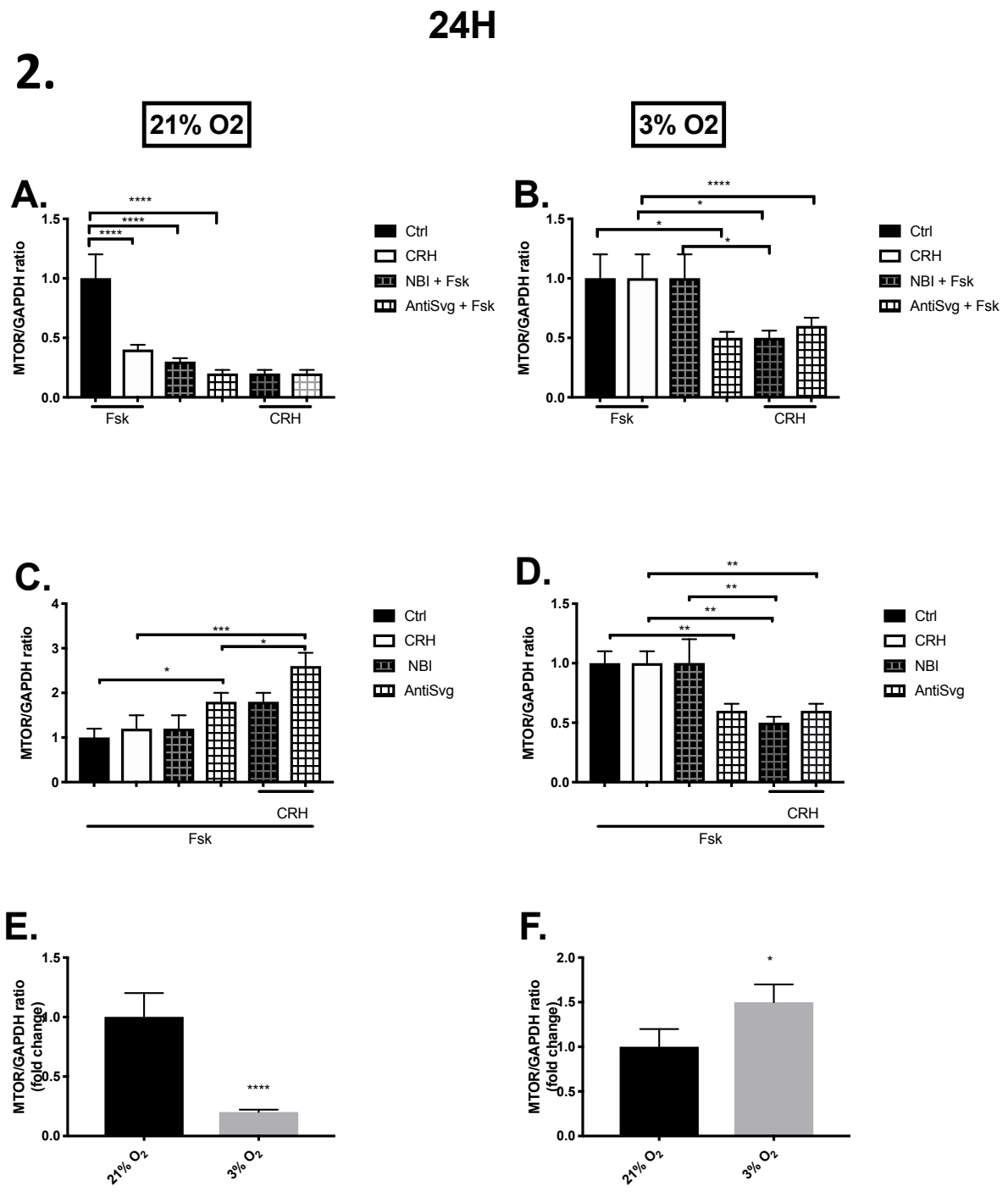


Figure 61

a. *Crh decreases mtor expression in bewo syncytialised cells. Crh has no effect on mtor expression on Blocking crhr1 or crhr2.*

b. *Crh has no effect on mtor expression in bewo syncytialised cells. Blocking crhr2 reduces mtor expression in bewo syncytialised cells in hypoxia. Crh reduces mtor expression on blocking crhr1 but not crhr2 in bewo syncytialised cells.*

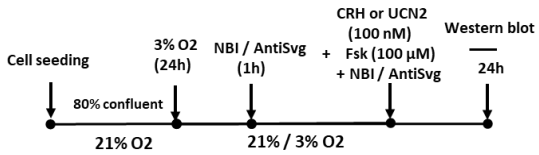
c. *crh has no effect on mtor expression in bewo syncytialised cells. Blocking crhr 1 has no effect, but blocking crhr2 increases mtor expression in bewo syncytialised cells. Crh increases mtor expression on blocking crhr2 in bewo syncytialised cells.*

Hypoxia decreases mtor expression at 21% O2 but increases at 3% o2.

Table 31

24H	MTOR	
	21% O2	3% O2
Ctrl	NS	↓
Fsk	NS	NS
CRH + Fsk	↓	NS
NBI + Fsk	↓	NS
AntiSvg + Fsk	↓	↓
CRH + NBI + Fsk	NS	↓
CRH + AntiSvg + Fsk	NS	NS

1.



2.

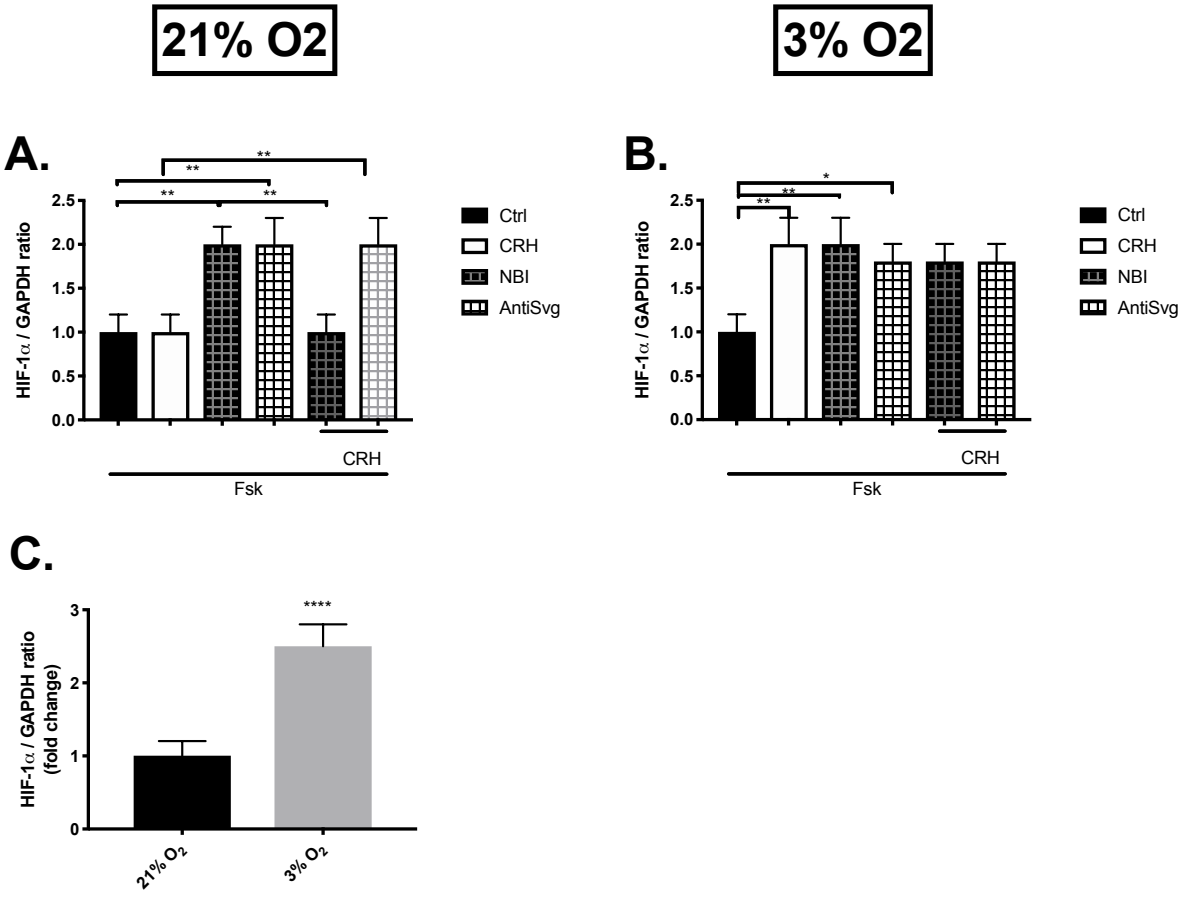


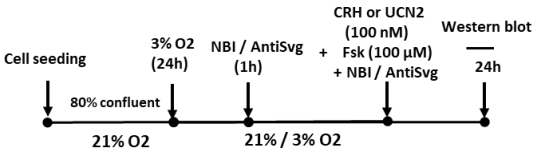
Figure 62

crh has no effect on hif-1a expression at 21% o2, but increases 3% o2 in bewo syncytialised cells. Blocking crhr1 or crhr2 increases hif-1a expression in bewo syncytialised cells. Crh has no effect on hif-1a expression in bewo syncytialised cells on blocking crhr1. Crh increases hif-a expression on blocking crhr2 in bewo syncytialised cells. At 3% o2, blocking crhr1 or crhr2 increases HIF-1a expression in bewo syncytialised cells. Crh has no effect on hif -1 a expression on blocking crhr1 or crhr2 in bewo syncytialised cells. Hypoxia increase hif-1a expression.

Table 32

24H	**HIF-1a**	
	21%O2	**3%O2**
Ctrl	NS	↑
Fsk	NS	NS
CRH + Fsk	NS	↑
NBI + Fsk	↑	↑
AntiSvg + Fsk	↑	↑
CRH+ Fsk + NBI	NS	NS
CRH + Fsk + AntiSvg	NS	NS

1.


Cell seeding
3% O2
(24h)
NBI / AntiSvg
(1h)
+
CRH or UCN2
(100 nM)
Fsk (100 µM)
+ NBI / AntiSvg
Western blot
24h
80% confluent
21% O2
21% / 3% O2

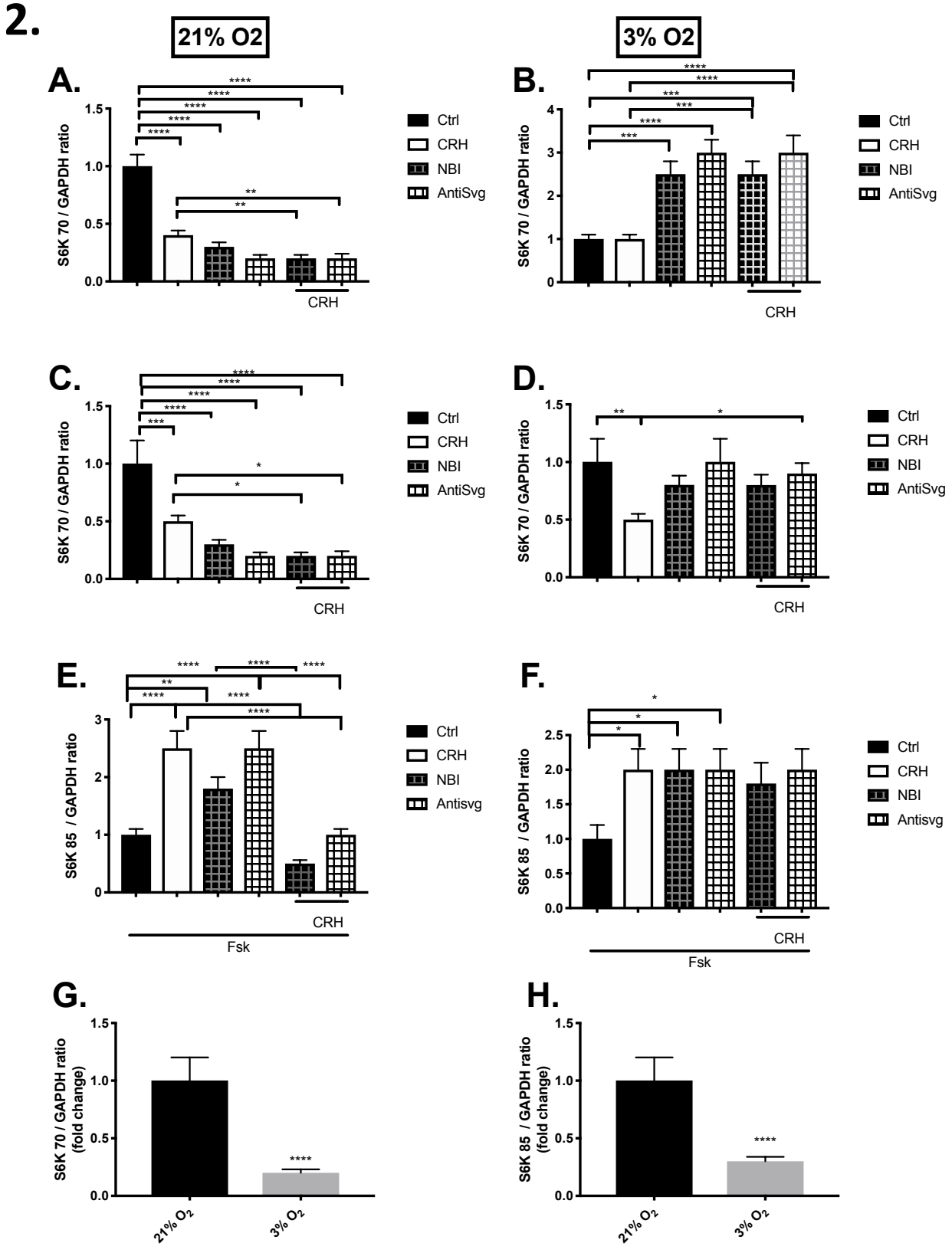


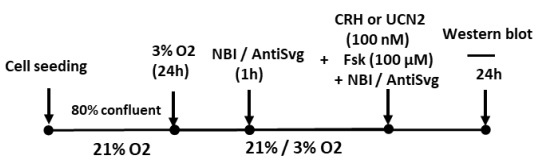
Figure 63

hypoxia decreases s6k70 and s6k85 expression. Crh decreases s6k70 expression at 21% o2 , but has no effect on s6k 70 expression at 3% o2. Crh has no effect on s6k70 expresssion (but in d shows decrease) on blocking crhr1 or crhr2 at 21 % o2 and 3% o2. Crh increases s6k85 expression in bewo syncytialised cells at both 21 % o2 and 3% o2. Blocking crhr1 or crhr2 increases s6k85 expression in bewo syncytialised cells at 21% o2 but not 3% o2. Crh decreases s6k85 expression at 21% o2 but no effect on s6k85 expression at 3% o2 on blocking crhr1 or crhr2 in bewo syncytialised cells.

Table 33

	S6k70		S6K85	
	21% O2	**3% O2**	**21% O2**	**3% O2**
Ctrl	NS	↓	NS	↓
CRH	↓	↓	NS	NS
NBI	↓	↑	NS	NS
AntiSvg	NS	↑	NS	NS
CRH + NBI	↓	NS	NS	NS
CRH + AntiSvg	↓	NS	NS	NS
Fsk	NS	NS	NS	NS
CRH + Fsk	NS	NS	↑	↑
Fsk + NBI	NS	NS	↑	↑
Fsk + AntiSvg	NS	NS	↑	↑
CRH + Fsk + NBI	NS	NS	↓	NS
CRH + Fsk + AntiSvg	NS	NS	↓	NS

1.



2.

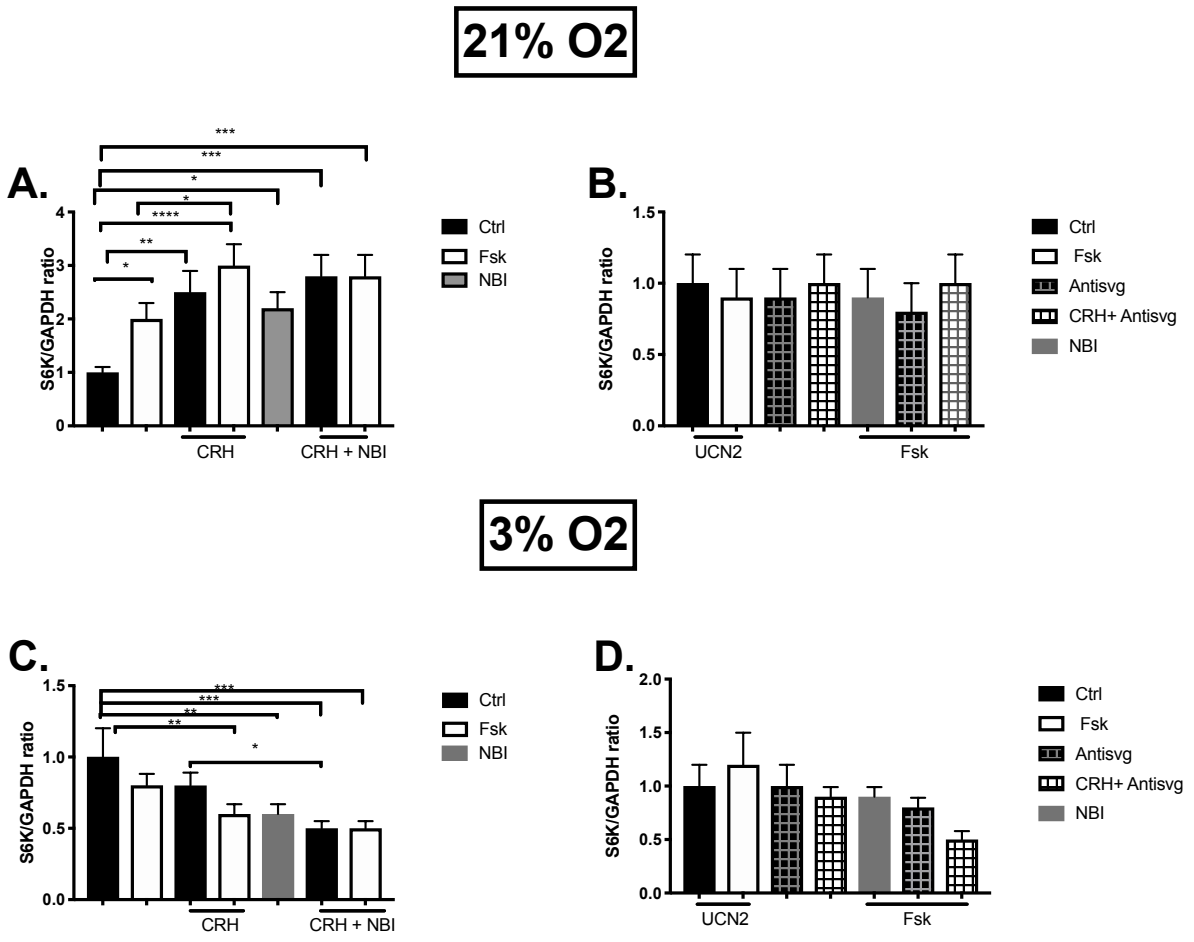


Figure 64

a. *Forskolin increases s6k expression. Crh increases s6k expression in both unsyncytialised and syncytialised bewo cells.*
Crh has no effect on s6k expression on blocking crhr1 in both unsyncytialised and syncytialised bewo cells

b. *Hypoxia blocks the effect of forskolin and crh on s6k expression. Hypoxia blocks the effect of crh on s6k expression in bewo syncytialised cells. Crh decreases s6k expression on blocking crhr1.*

Table 34

S6K/GAPDH ratio (24H)	**21%O2**	**3% O2**
Ctrl	NS	NS
Fsk	↑	NS
CRH	↑	NS
CRH + Fsk	↑	NS
NBI	↑	NS

CRH + NBI	NS	NS
CRH + NBI + Fsk	NS	NS
UCN2	NS	NS
UCN2 + Fsk	NS	NS
AntiSvg	NS	NS
CRH + AntiSvg	NS	NS
Fsk + NBI	NS	NS
Fsk + AntiSvg	NS	NS
CRH + AntiSvg + Fsk	NS	NS

Bewo 15’

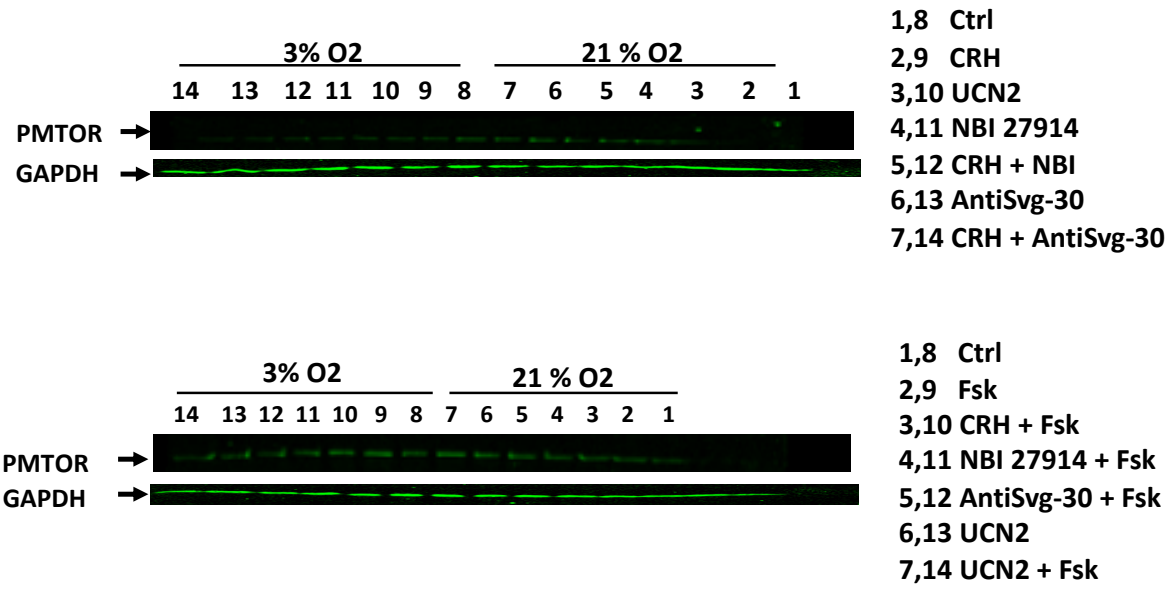
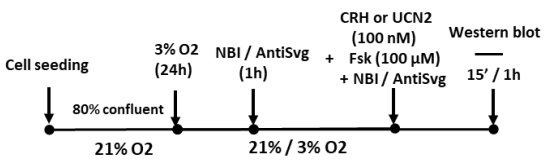


Figure 65

1.



2.

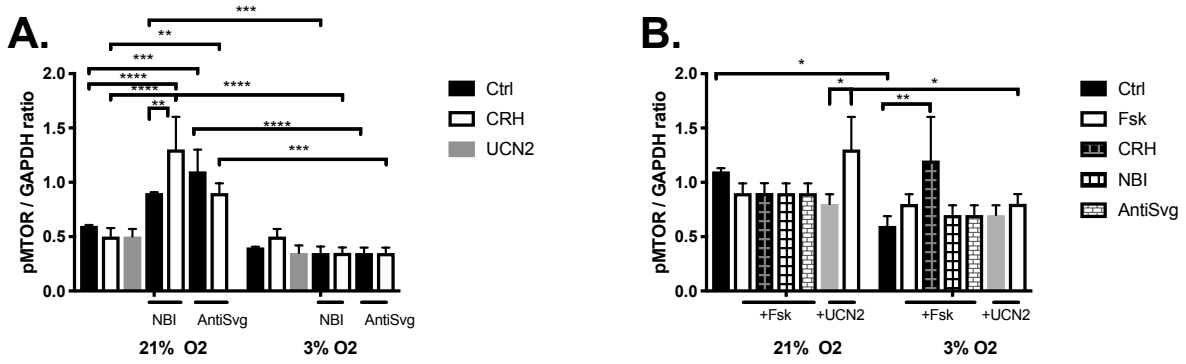


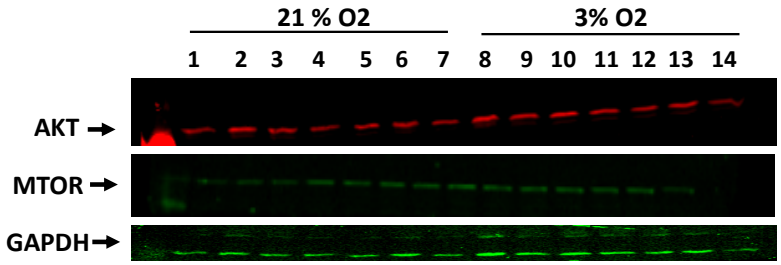
Figure 66

Crh and ucn2 has no effect on pmtor at 21 and 3% o2. Crh increases pmtor on blocking crhr1 but not crhr2. At 3% O2, Crh has no effect on pmtor on blocking crhr1 or crhr2. Forskolin has no effect on pmtor at 21 and 3% o2. Crh has no effect on pmtor in bewo syncytialised cells at 21% o2 but increases pmtor at 3% o2. Blocking crhr1 or crhr2 does not affect pmtor in bewo syncytialised cells at 21% o2 and 3% o2. Ucn2 has no effect on pmtor at 21 and 3 % o2 in bewo cells. Ucn2 increases pmtor in bewo syncytialised cells at 21% o2 but not 3% o2.

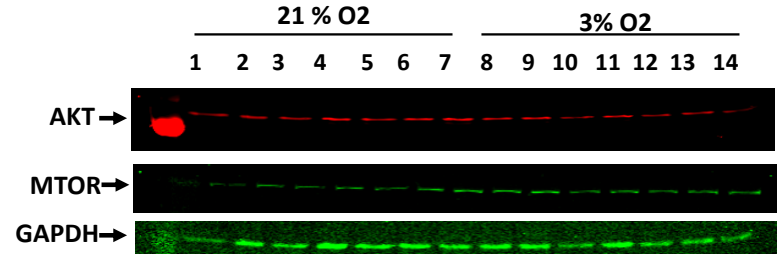
Table 35

15'	PMTOR		PS6K	
	21%O2	3%O2	21%O2	3%O2
Ctrl	NS	↓	NS	NS
CRH	NS	NS	NS	NS
UCN2	NS	NS	NS	NS
NBI	NS	NS	NS	NS
CRH + NBI	↑	NS	NS	NS
AntiSvg	NS	NS	NS	NS
CRH+AntiSvg	NS	NS	NS	NS
Fsk	NS	NS	NS	NS
CRH + Fsk	NS	NS	NS	NS
Fsk + NBI	NS	NS	NS	NS
Fsk + AntiSvg	NS	NS	NS	NS
UCN2	NS	NS	NS	NS
UCN2 + Fsk	↑	NS	NS	NS

Bewo 1h



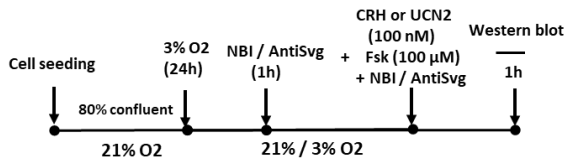
1,8 Ctrl
2,9 CRH
3,10 Fsk
4,11 NBI 27914
5,12 CRH + NBI 27914
6,13 AntiSvg-30
7,14 CRH + AntiSvg-30



1,8 Fsk
2,9 CRH + Fsk
3,10 UCN2 + Fsk
4,11 NBI 27914 + Fsk
5,12 AntiSvg-30 + Fsk
6,13 CRH + NBI 27914 + Fsk
7,14 CRH + AntiSvg-30 + Fsk

Figure 67

1.



2.

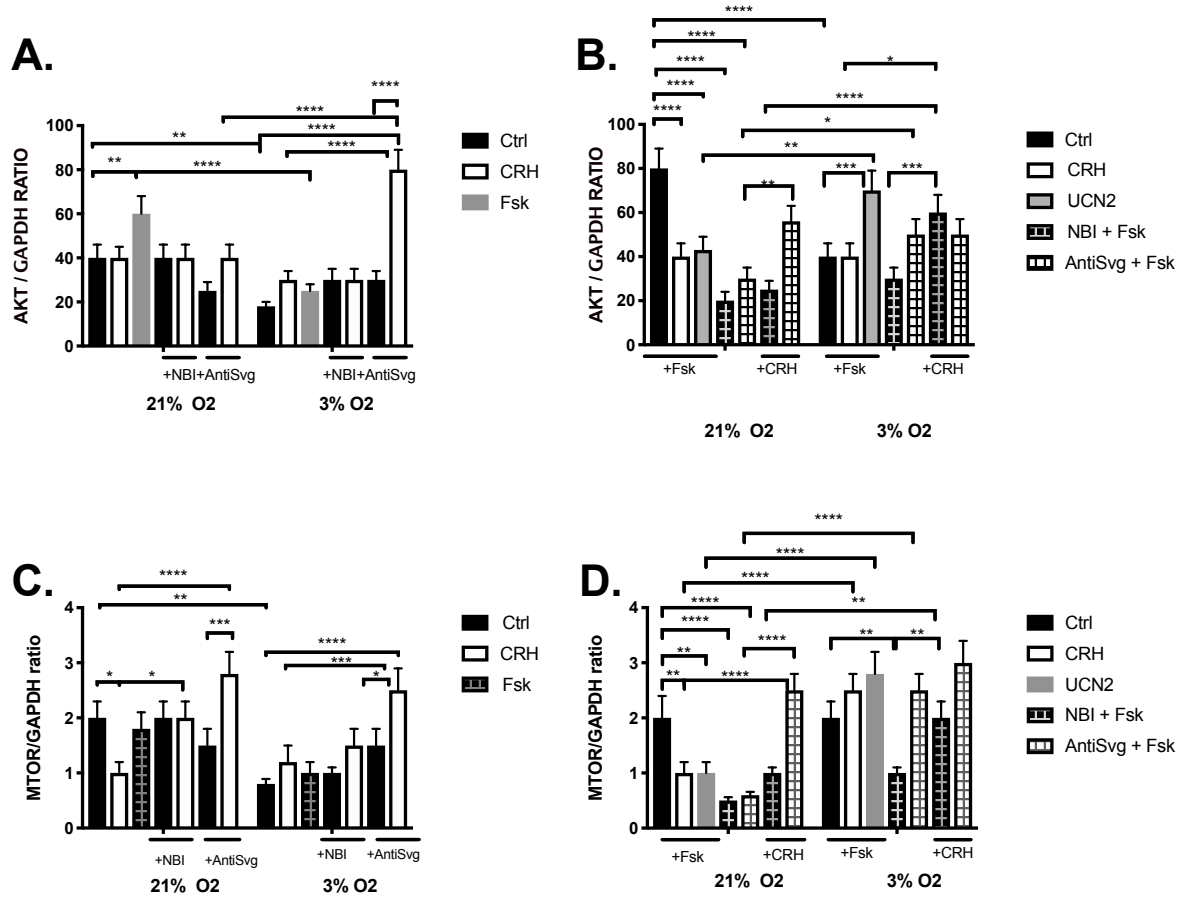


Figure 68

Hypoxia decreases akt expression. CRH has no effect on akt expression at 21% o2 and 3% o2. Forskolin increases akt expression and hypoxia blocks it. Blocking crhr1 has no effect on akt expression in bewo syncytialised cells at 21% o2. Blocking crhr2 increases akt expression at 3% o2, but not at 21% o2 in bewo syncytialised cells. crh and ucn2 reduces akt expression in bewo syncytialised cells. Crh increases akt expression on blocking crhr2. Crh has no effect but ucn2 increases akt expression in bewo syncytialised cells. Crh increases akt expression in bewo syncytialised cells on blocking crhr1. Crh reduces mtor expression and hypoxia blocks it. Forskolin has no effect on mtor expression in both 21 and 3% o2. Crh has no effect on mtor expression on blocking crhr1. Crh increases mtor expression on blocking crhr2. Crh and ucn2 decreases mtor expression in bewo syncytialised cells at 21% o2. Crh increases mtor expression on blocking crhr2. Hypoxia – crh and ucn2 has no effect on mtor expression in bewo syncytialised cells. Crh increases mtor expression on blocking crhr1 but no effect on mtor expression on blocking crhr2.

Table 36

1H	**AKT**		**MTOR**	
	21%O2	3%O2	21%O2	3%O2
Ctrl	NS	↓	NS	↓
CRH	NS	NS	↓	NS
UCN2	NS	NS	NS	NS
Fsk	↑	NS	NS	NS
NBI	NS	NS	NS	NS

CRH + NBI	NS	NS	NS	NS
AntiSvg	NS	NS	NS	NS
CRH + AntiSvg	NS	↑	↑	↑
Fsk	NS	NS	NS	NS
CRH + Fsk	NS	↓	↓	NS
UCN2 + Fsk	NS	↓	↓	NS
NBI + Fsk	NS	↓	NS	↓
AntiSvg + Fsk	NS	↓	NS	NS
CRH + NBI + Fsk	NS	NS	NS	↑
CRH + AntiSvg + Fsk	NS	↑	↑	NS

Bewo 1h

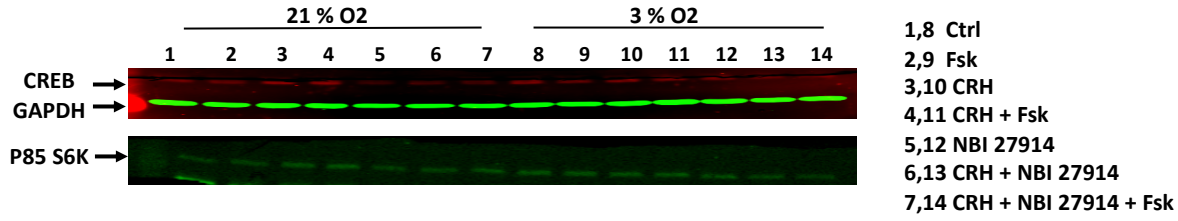
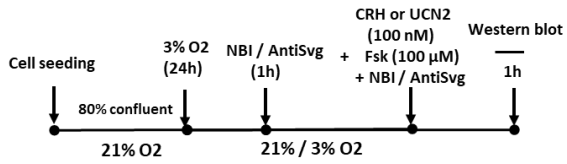


Figure 69

1.



2.

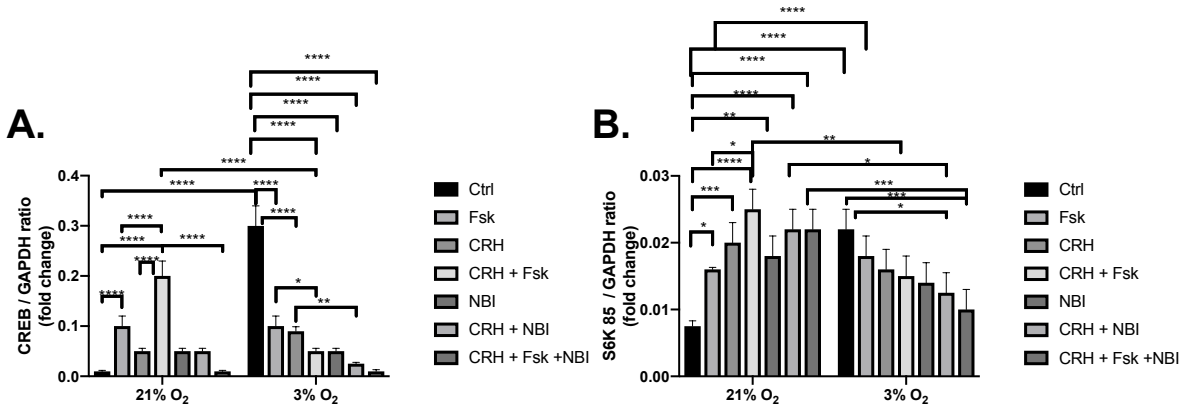


Figure 70

hypoxia increases creb expression. Hypoxia reverses the effect of forskolin and crh on creb expression. Hypoxia reverses th crh effect on creb expression in bewo syncytialised cells at 3% o2, crh has no effect on creb expression on blocking crhr1. Crh has no effect on creb expression in bewo syncytialised cells on blocking crhr1. Hypoxia blocks the increased s6k85 expression induced by forskolin and crh. Hypoxia blocks increased s6k 85 expression mediated by crh in bewo syncytialised cells. Crh has no effect on s6k85 expression on blocking crhr1 in unsyncytialised and syncytialised bewo cells at 21 % O2 and 3% o2.

Table 37

1H	**CREB**		**S6K85**	
	21%O2	3%O2	21%O2	3%O2
Ctrl	NS	↑	NS	↑
Fsk	↑	↓	↑	NS
CRH	NS	↓	↑	NS
CRH+Fsk	↑	↓	↑	NS
NBI	NS	↓	NS	↓
CRH + NBI	NS	NS	NS	NS
CRH + Fsk + NBI	↓	↓	NS	NS

Bewo 15’

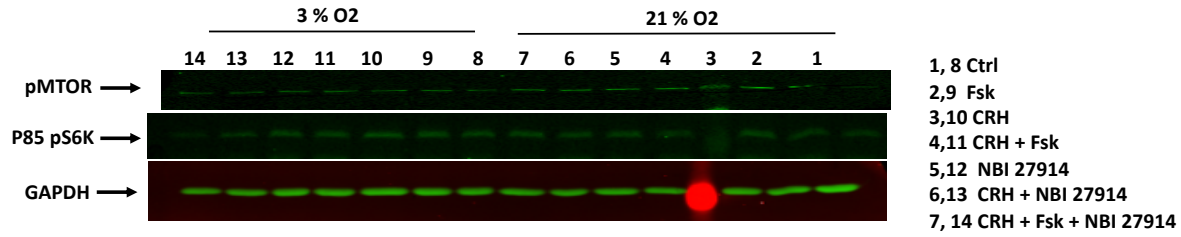


Figure 71

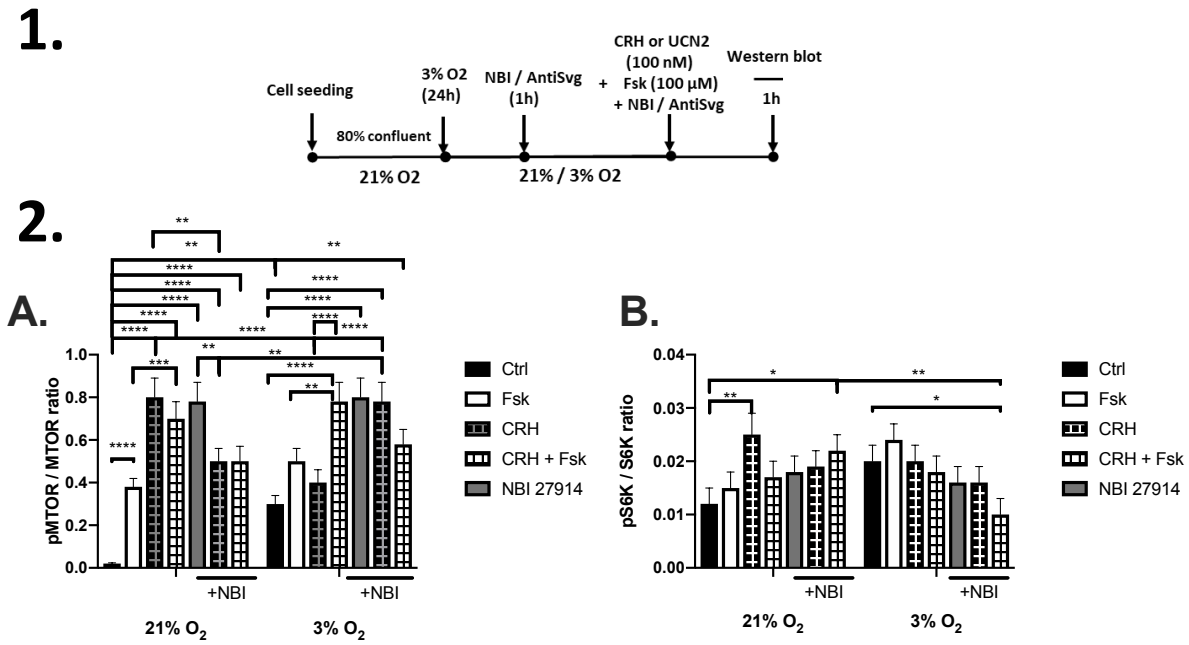


Figure 72

Forskolin and crh increases pmtor at 21% o2. Hypoxia blocks it. Crh increases pmtor in bewo syncytialised cells at 21% and 3% o2. Crh reduces pmtor on blocking crhr1. Crh has no effect on pmtor on blocking crhr1 in bewo syncytialised cells. At 3% o2, Crh has no effect on pmtor on blocking crhr1 in bewo cells and unsyncytialised cells.

Table 38

15’	PMTOR		PS6K	
	21%O2	3%O2	21%O2	3%O2
Ctrl	NS	↑	NS	NS
Fsk	↑	NS	NS	NS

CRH	↑	NS	↑	NS
CRH+Fsk	↑	↑	NS	NS
NBI	↑	↑	NS	NS
CRH + NBI	↓	↑	NS	NS
CRH + Fsk + NBI	↓	NS	NS	NS

Bewo 24h

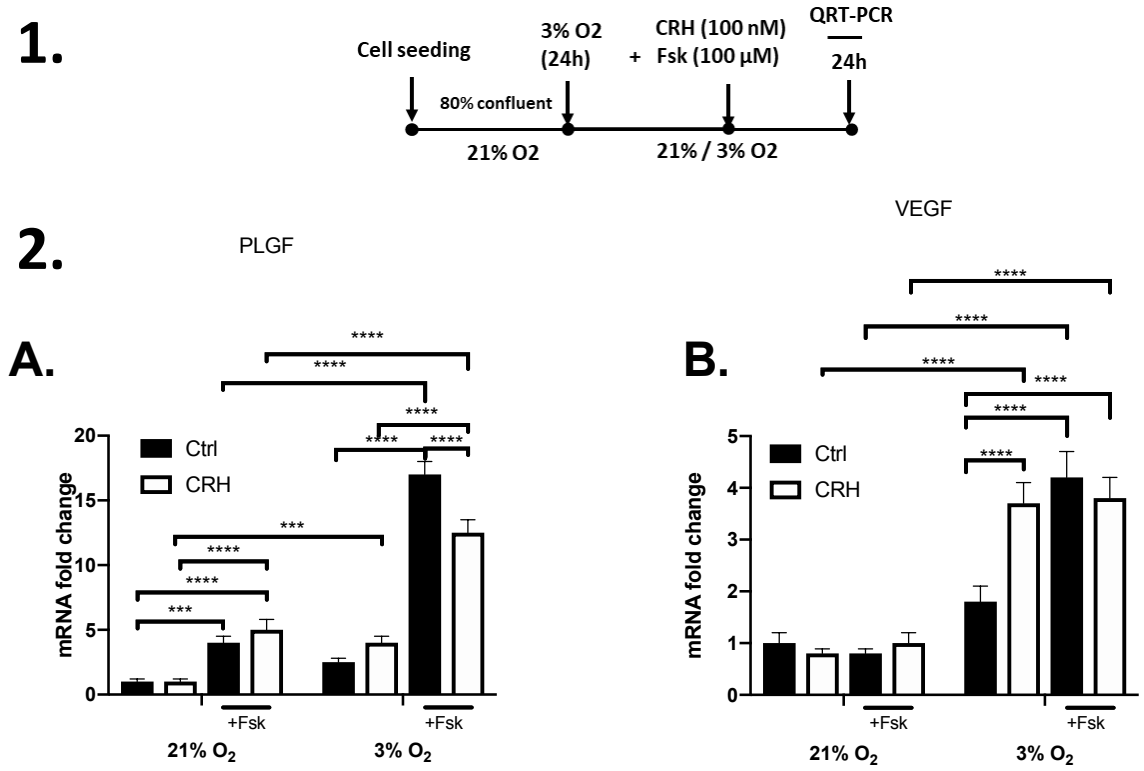


Figure 73

Table 39

MRNA	**PLGF**		**VEGF**	
	21% O2	**3% O2**	**21% O2**	**3% O2**
Ctrl	NS	NS	NS	NS
Fsk	↑	↑	NS	↑
CRH	NS	NS	NS	↑
CRH + Fsk	↑	↓	NS	↑

Bewo 15’

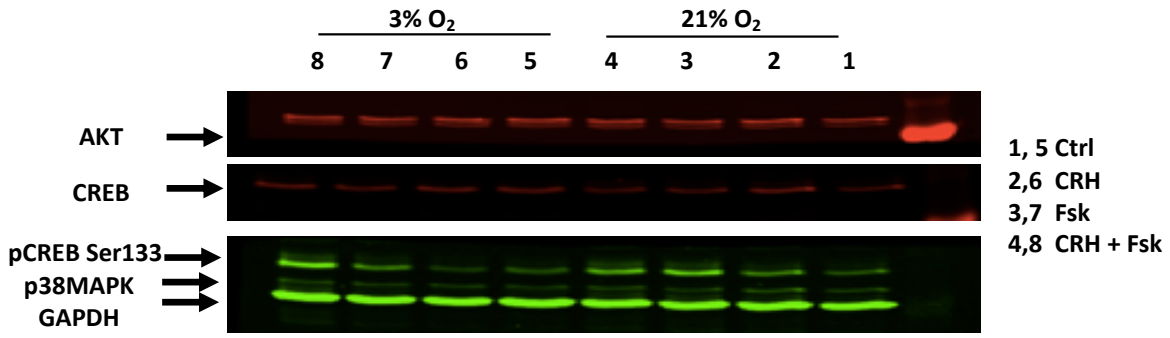


Figure 74

Bewo 24h

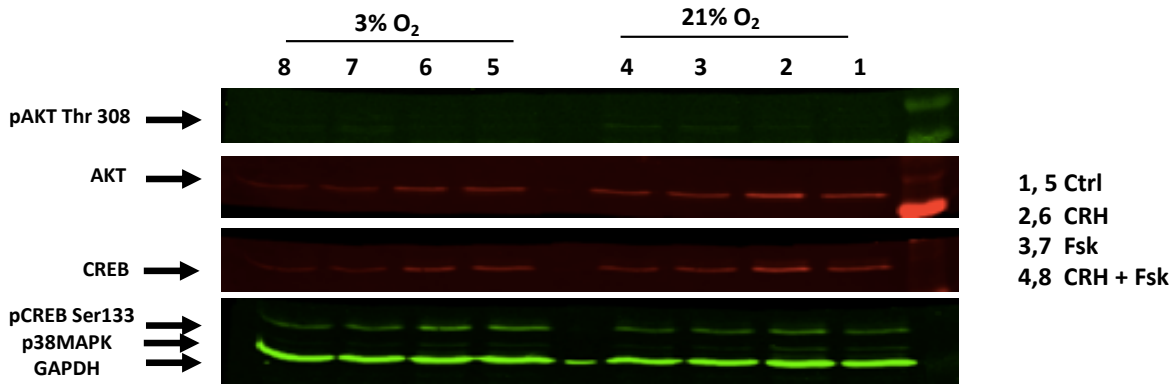


Figure 75

Bewo 24h

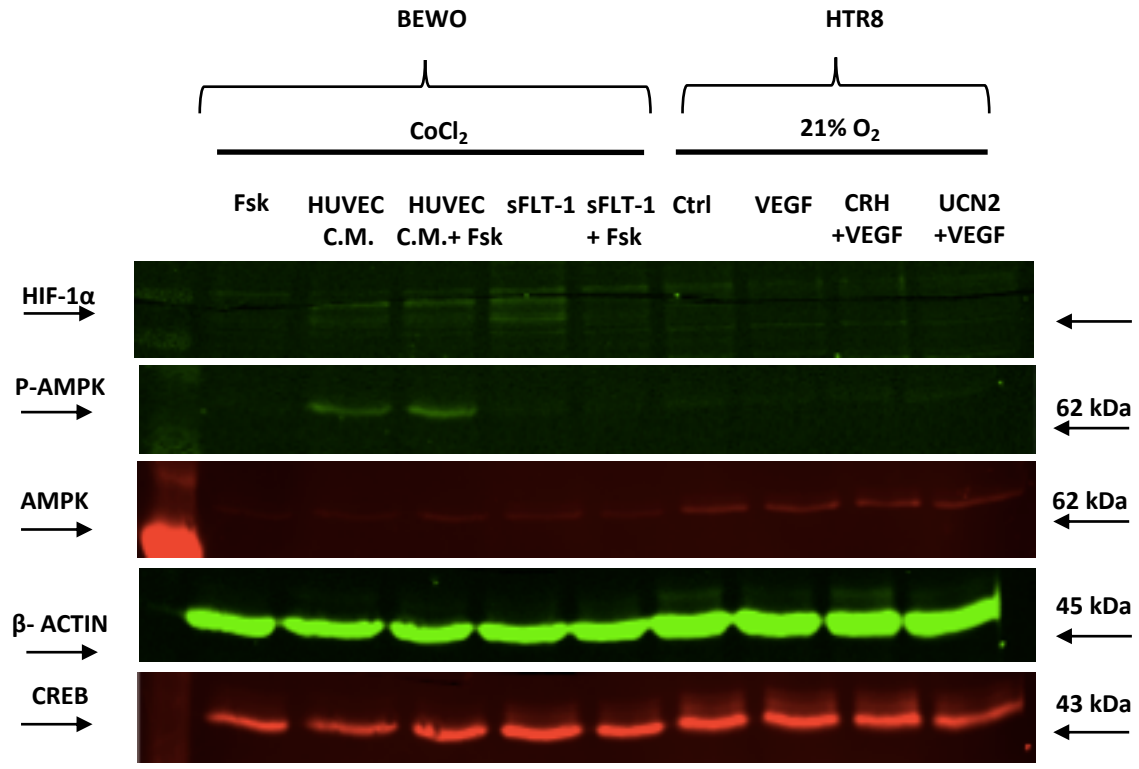


Figure 76

Bewo 15'

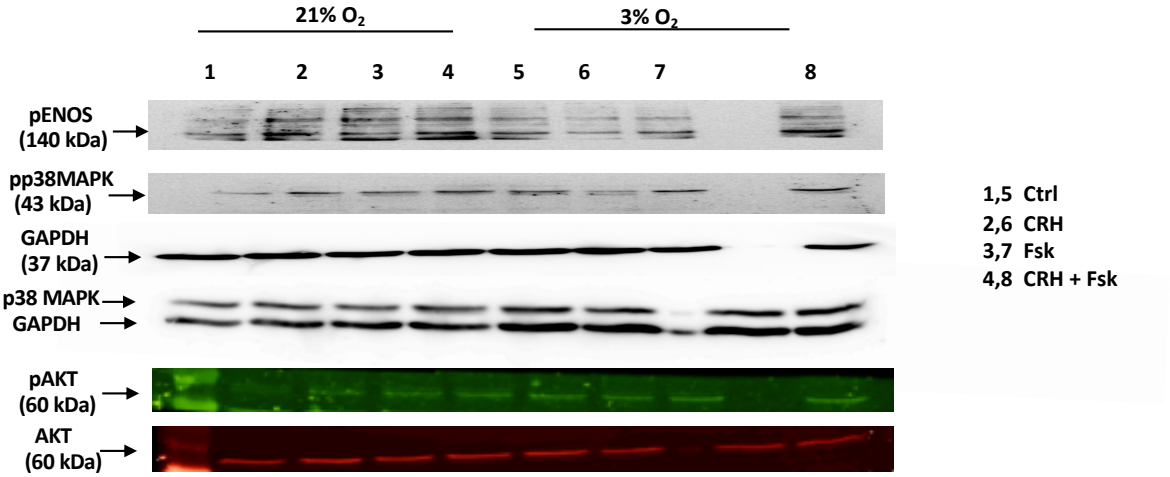


Figure 77

Bewo 15’

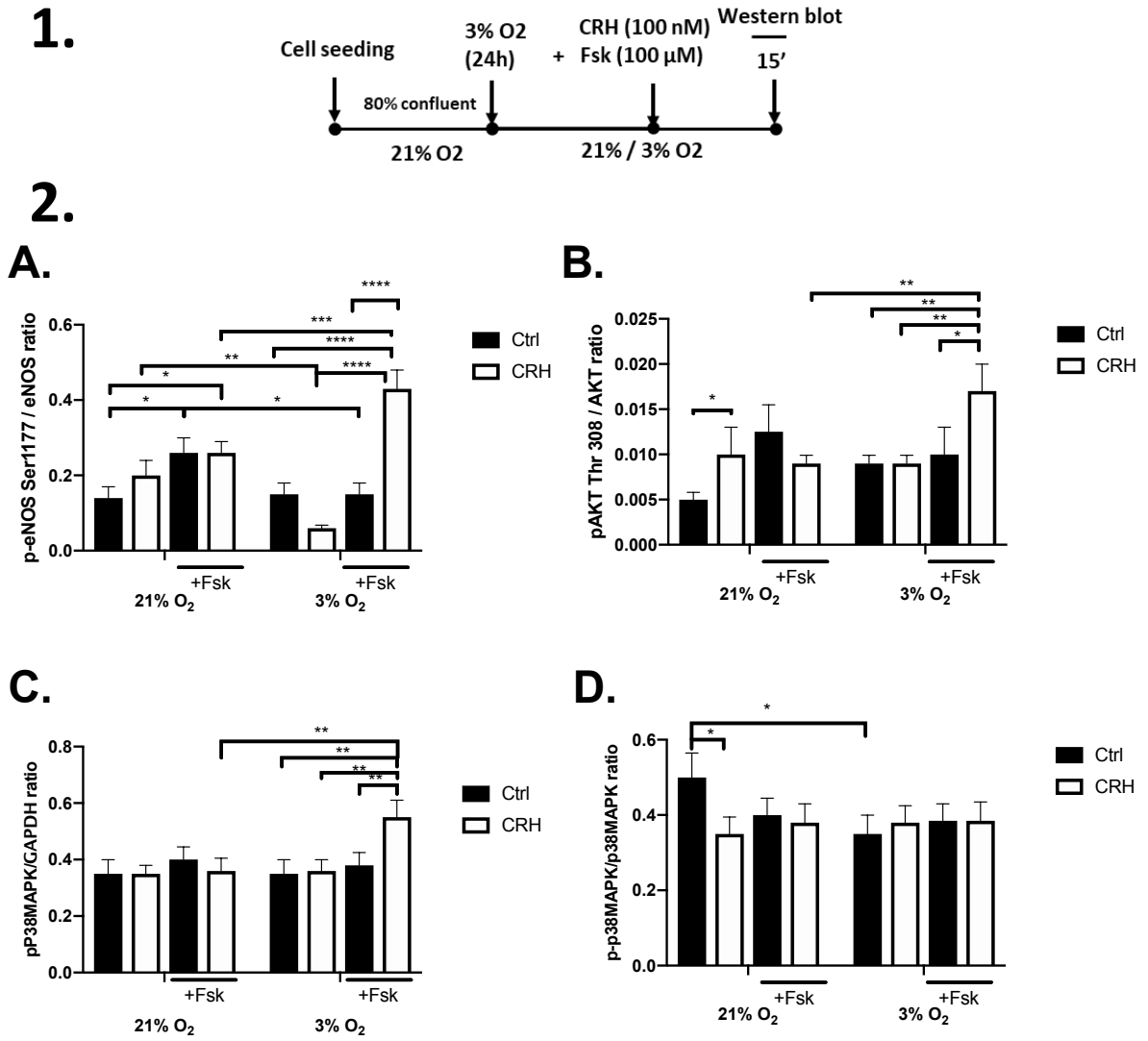


Figure 78

Crh has no effect on p-enos at 21 and 3% 02. Forskolin increases penos at 21% o2 but no effect on penos at 3% o2. Crh has no effect on penos at 21% o2 but increases penos at 3% o2 in bewo syncytialised cells. Crh increases pakt at 21% o2 but lost at 3% o2. Crh increases pakt at 21% o2 but blocked at low oxygen tension. Crh has no effect on pakt at 21% o2 but increases at 3% o2 in bewo syncytialised cells. Crh and forskolin has no effect on Pp38 mapk expression at 21 and 3% o2. Crh has no effect on pp38 mapk expression in bewo syncytialised cells at 21% o2 but increases pp38 mapk at 3% o2.

Table 40

	21% O2				3% O2			
15'	**Ctrl**	**CRH**	**Fsk**	**CRH + Fsk**	**Ctrl**	**CRH**	**Fsk**	**CRH + Fsk**
PPENOS	NS	NS	↑	NS	NS	NS	NS	↑
PAKT THR308	NS	↑	NS	NS	NS	NS	NS	↑
PP38 MAPK / GAPDH ratio	NS	NS	NS	NS	NS	NS	NS	↑
PP38 MAPK / P38 MAPK ratio	NS	↓	NS	NS	↓	NS	NS	NS

Bewo 24h

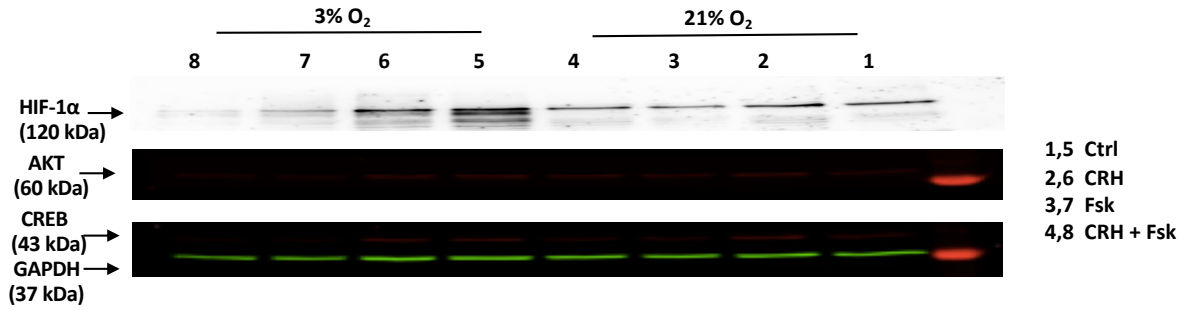


Figure 79

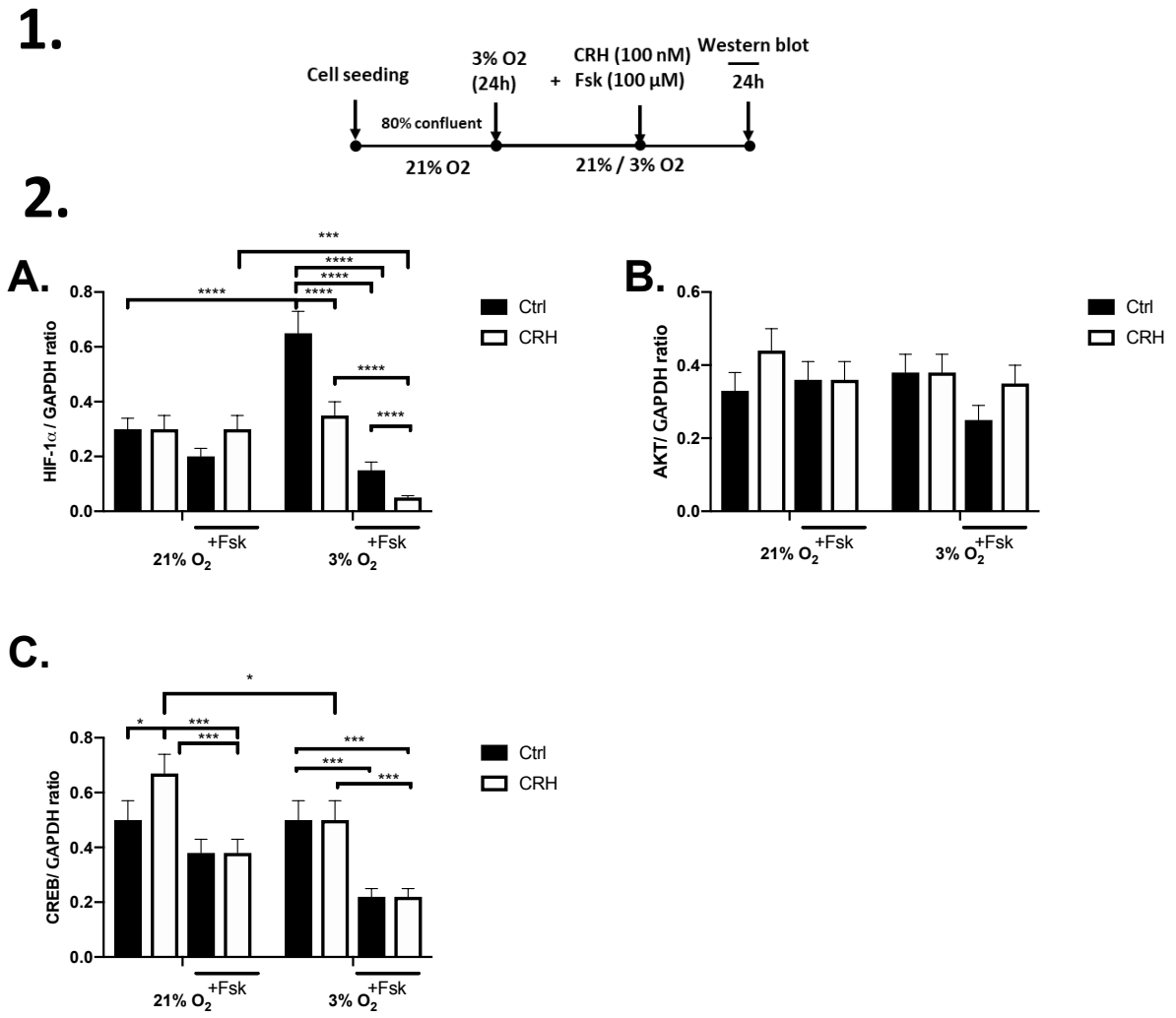


Figure 80

crh and forskolin has no effect on hif-1a expresion at 21% o2 but reduces hif-1a expression at 3% o2. Crh has no effect on hif-1a expression in syncytialised bewo cells at 21% o2 but decreases at 3% o2. Forskolin has no effect on akt expression at 21% o2 and3% o2. Crh has no effect on akt expression in bewo syncytialised cells at 21% o2 and 3% o2. Crh increases creb expression at 21% o2 but hypoxia blocks it. Forskolin has no effect on creb expression but reduces creb expression at 3% o2. Crh has no effect on creb expression in bewo syncytialised cells at 21 and 3% o2.

Table 41

	21% O2				**3% O2**			
24H	**Ctrl**	**CRH**	**Fsk**	**CRH + Fsk**	**Ctrl**	**CRH**	**Fsk**	**CRH + Fsk**
HIF-1a	NS	NS	NS	NS	↑	↓	↓	↓
AKT	NS	NS	NS	NS	NS	NS	NS	NS
CREB	NS	↑	NS	↓	NS	NS	↓	NS

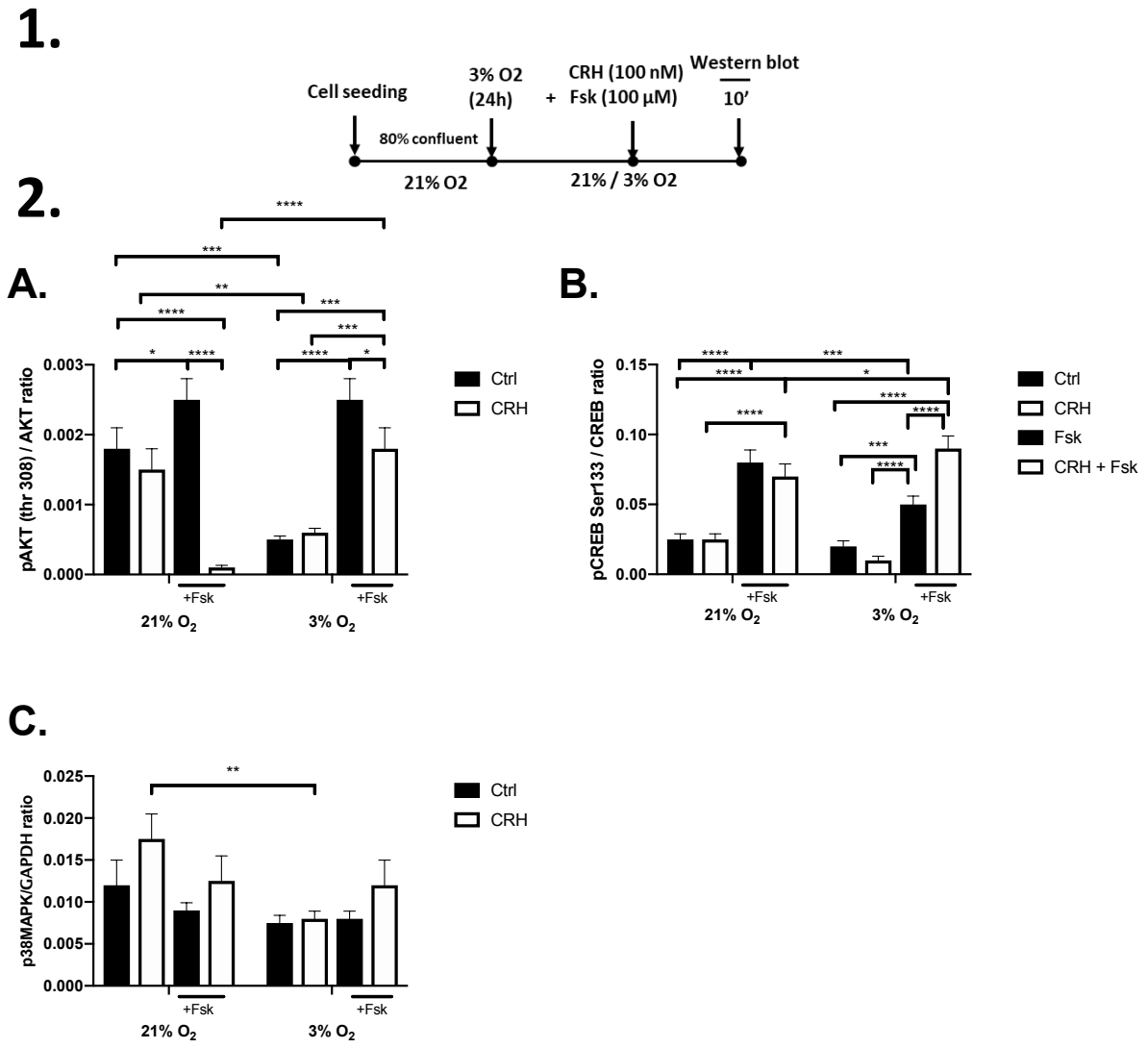


Figure 81

Hypoxia decreases pakt. Crh has no effect on pakt at 21 and 3% o2. Forskolin increases pakt and hypoxia potentiates it. Crh decreases pakt in bewo syncytialised cells and hypoxia reverses it. Crh has no effect on pcreb at 21 and 3% o2. Forskolin increases pcreb at 21 and 3% o2. Crh increases pcreb in bewo syncytialised cells at 21% o2 and hypoxia potentiates it. Crh and forskolin has no effect on p38 mapk at 21 and 3% o2. Crh has no effect on p38 mapk in bewo syncytialised cells at 21 and 3% o2.

Table 42

	21% O2				**3% O2**			
15'	**Ctrl**	**CRH**	**Fsk**	**CRH + Fsk**	**Ctrl**	**CRH**	**Fsk**	**CRH + Fsk**
PAKT THR308	NS	NS	↑	↓	↓	NS	↑	↓

PCREB SER133	NS	NS	↑	NS	NS	NS	↑	↑
P38 MAPK	NS	NS	NS	NS	NS	NS	NS	NS

Bewo 24h

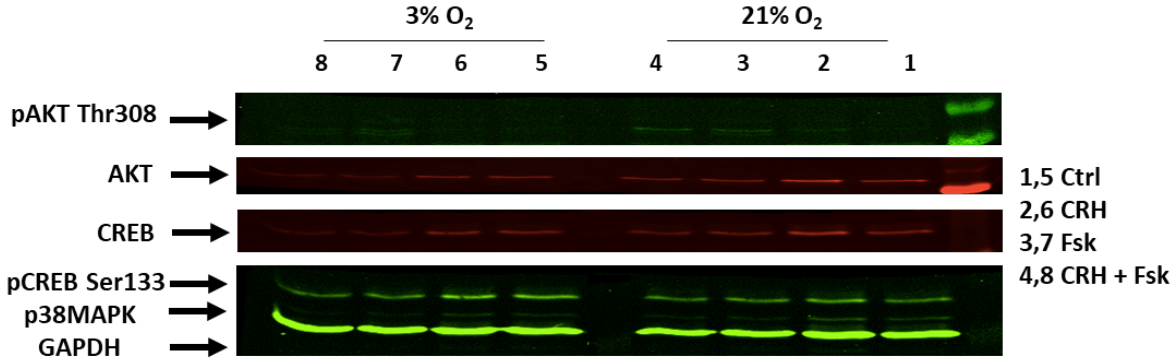
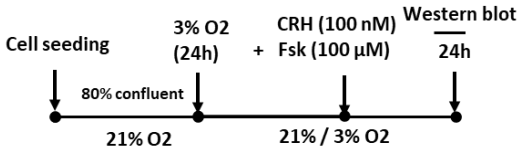


Figure 82

1.

2.



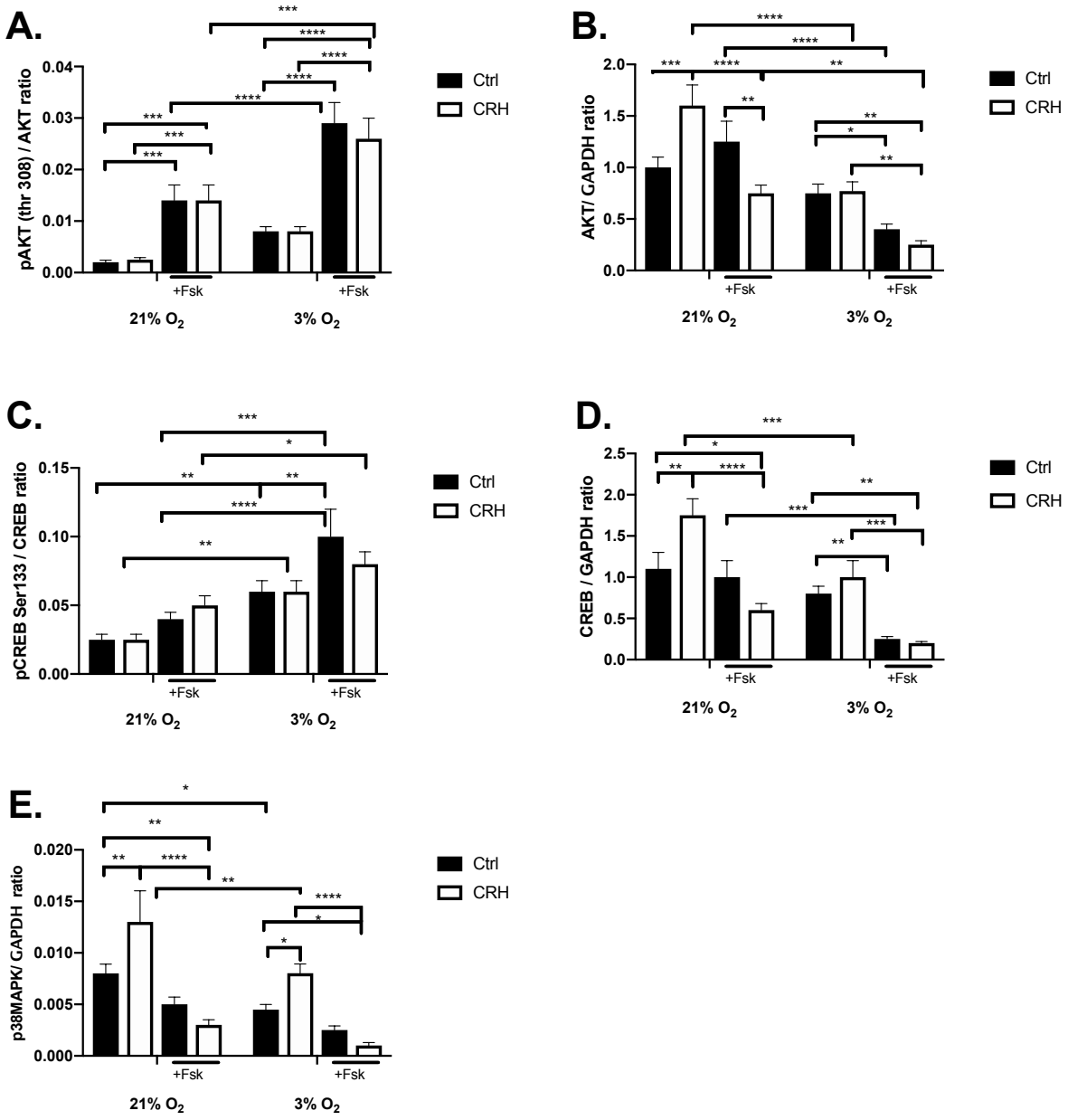


Figure 83

at 21% O2, crh increases and forskolin reduces phosphorylation of akt and ampk in bewo cells. Crh reduces pakt and pampk in bewo syncytialised cells.

Table 43

	21% O2				3% O2			
24H	**Ctrl**	**CRH**	**Fsk**	**CRH + Fsk**	**Ctrl**	**CRH**	**Fsk**	**CRH + Fsk**
PAKT THR308	NS	NS	↑	↑	↑	NS	↑	NS

AKT	NS	↑	NS	↓	NS	NS	↓	NS
PCREB SER133	NS	NS	NS	NS	NS	NS	NS	NS
CREB	NS	↑	NS	↓	NS	NS	↓	NS
P38 MAPK	NS	↑	NS	NS	↓	↑	NS	NS

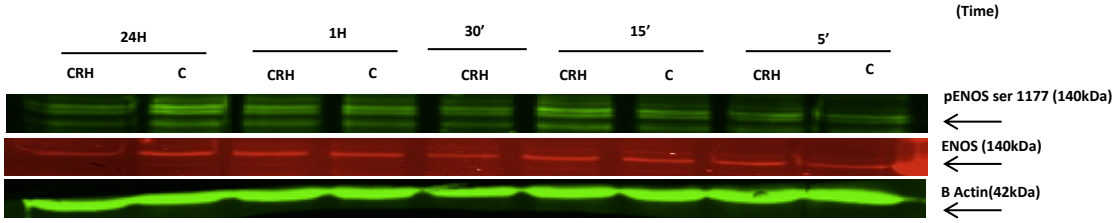


Figure 84

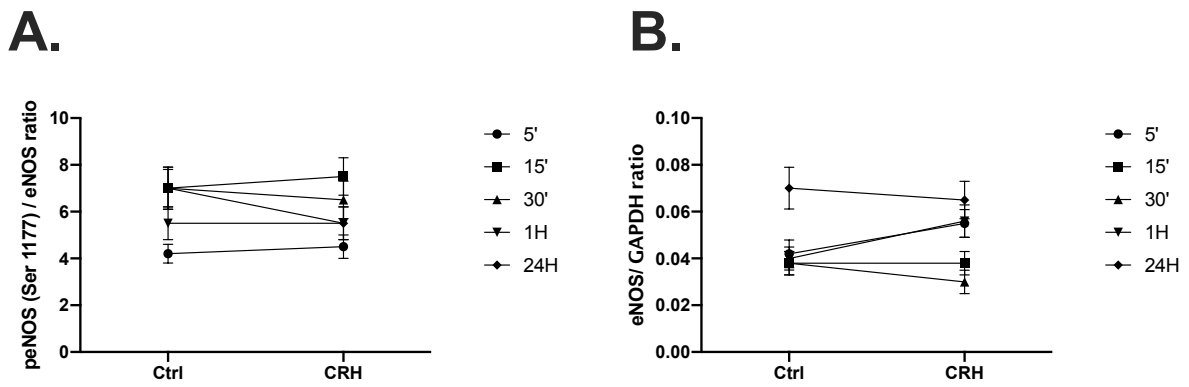


Figure 85

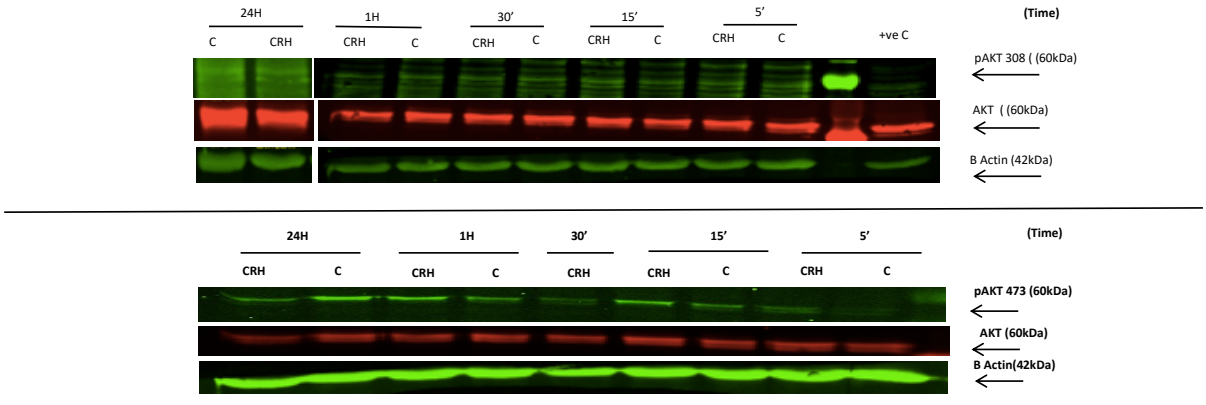


Figure 86

In BEWO cells, Penos increases at 15', 30' and 24h. Crh increases at 15'. Pakt 308 increases 15' at 21 and 3% o2. Akt decreases at 15'. Crh has no effect. Pakt ser 473 decreases at 1h. Crh no effect. Akt increases at 15; Crh increases akt at 5'and decreases at 5',30'.

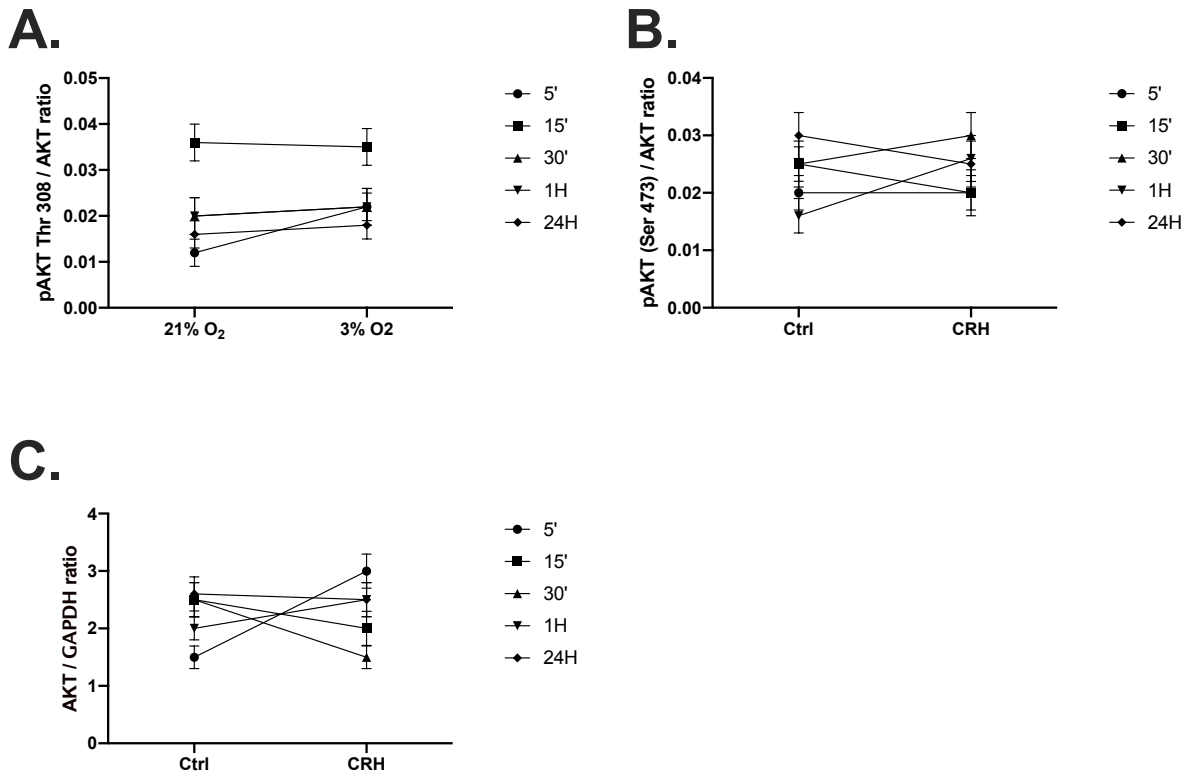


Figure 87

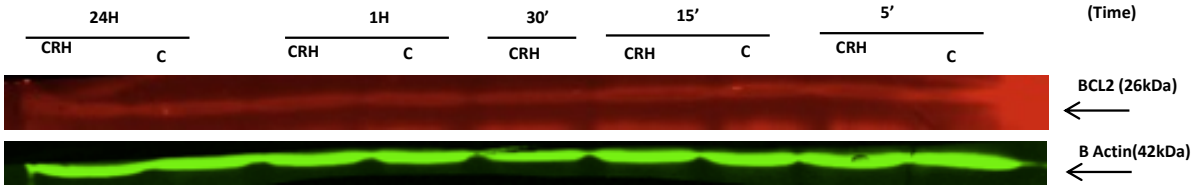


Figure 88

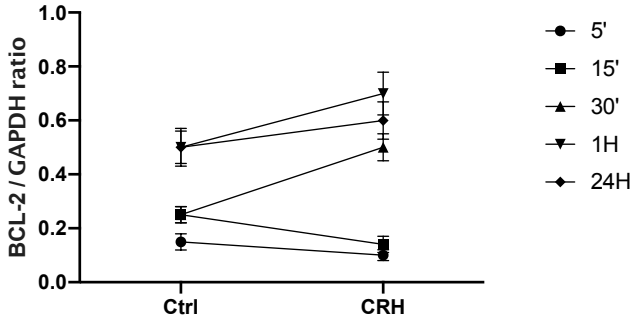


Figure 89

BCL2 increases at 1h in BEWO cells.

Table 44

	Ctrl					CRH				
	5'	**15'**	**30'**	**1H**	**24H**	**5'**	**15'**	**30'**	**1H**	**24H**
PENOS	NS	↑	↑	NS	↑	NS	↑	NS	NS	NS
ENOS	NS	NS	NS	NS	↑	NS	NS	↓	NS	NS
PAKT THR 308	NS	↑	NS	NS	NS	NS	↓	NS	NS	NS
PAKT SER 473	NS	NS	NS	↓	NS	NS	NS	NS	NS	NS
AKT	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
BCL2	NS	NS	NS	↑	↑	NS	NS	↑	↑	↑

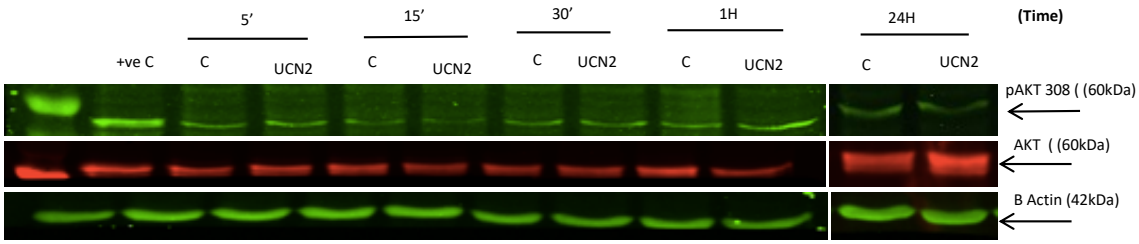


Figure 90

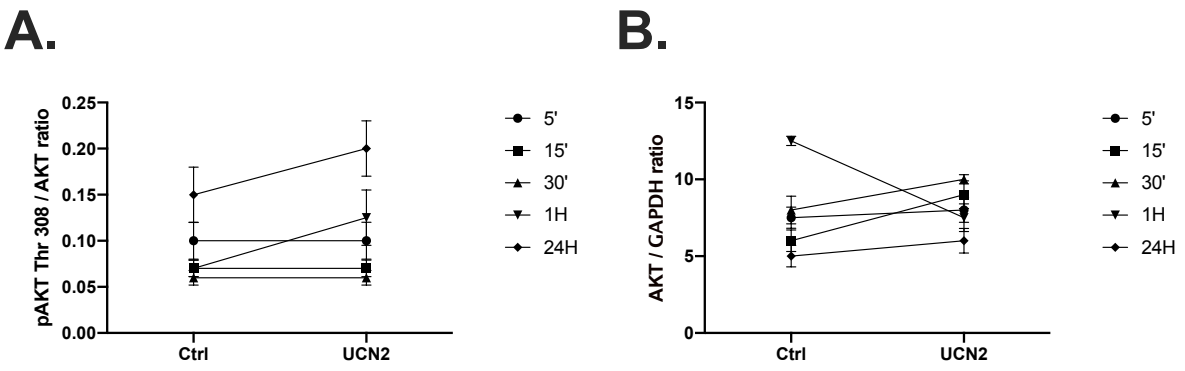


Figure 91

The effect of UCN2 on pAKTthr308 at different time points in BEWO cells.

Bewo 10’

P38 mapk , pcreb ns

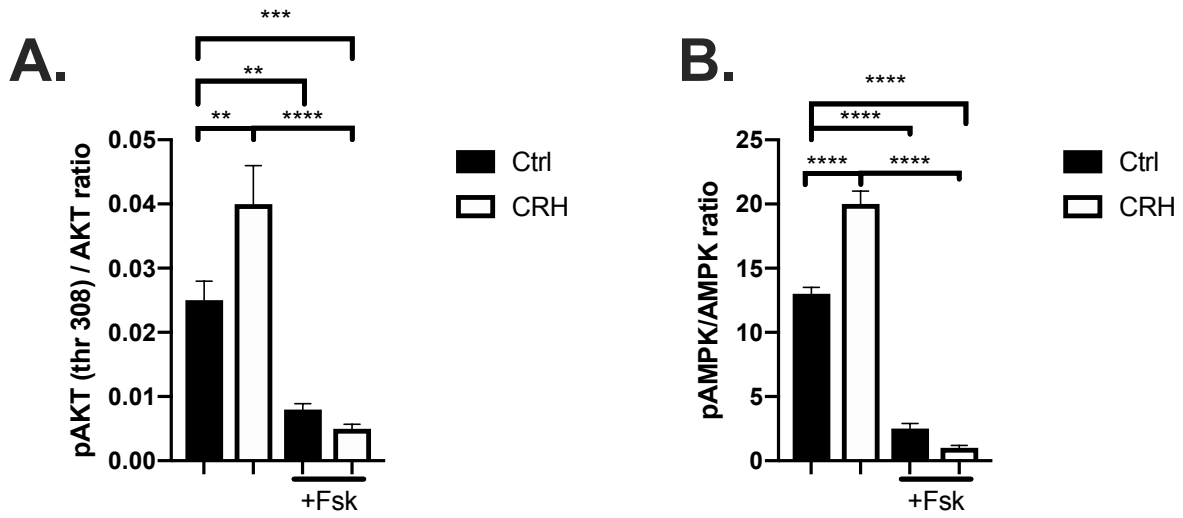


Figure 92

at 21% O2, crh increases and forskolin reduces phosphorylation of akt and ampk in bewo cells. Crh reduces pakt and pampk in bewo syncytialised cells.

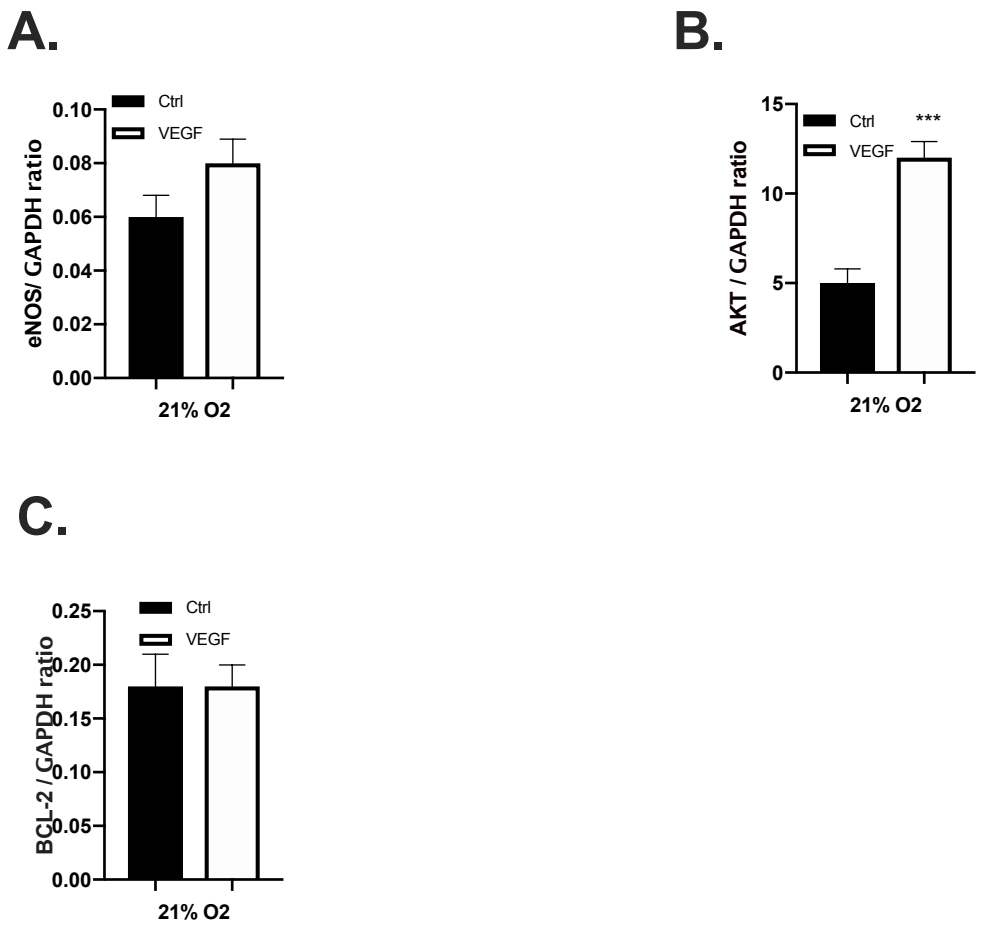


Figure 93

VEGF increases AKT expression in BEWO cells under high oxygen tension.eNOS and BCL2 expression remains unaffected.

6.1.20 The effect of hypoxia on the expression of fusogenic genes

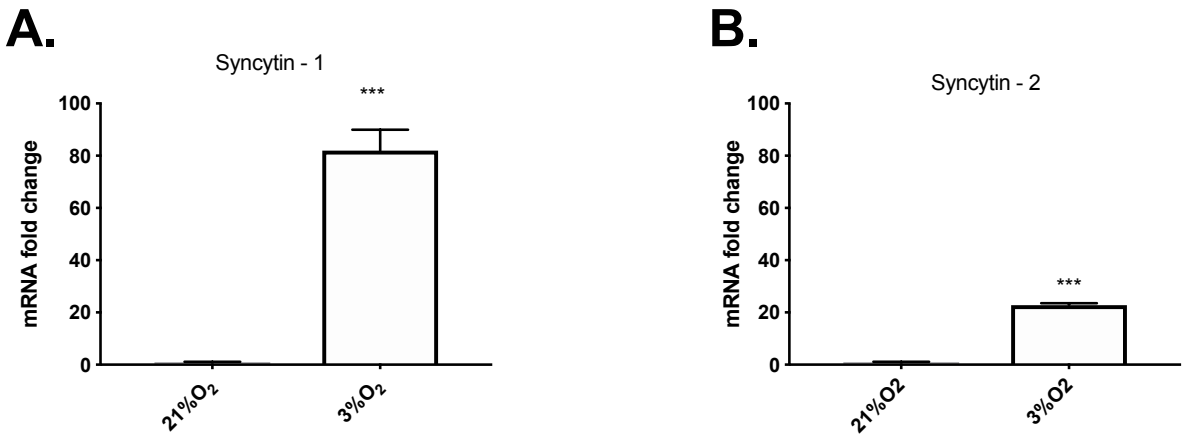


Figure 94

*Hypoxia increases syncytin 1 and 2 MRNA expression in BEWO cells. QRT PCR analysis showed that the expression of (A) syncytin 1 and (B) syncytin 2 mRNA was significantly upregulated for 24h at 3% O2. *** p=.0002; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.*

6.1.21 The expression of fusogenic genes in syncytialised bewo cells

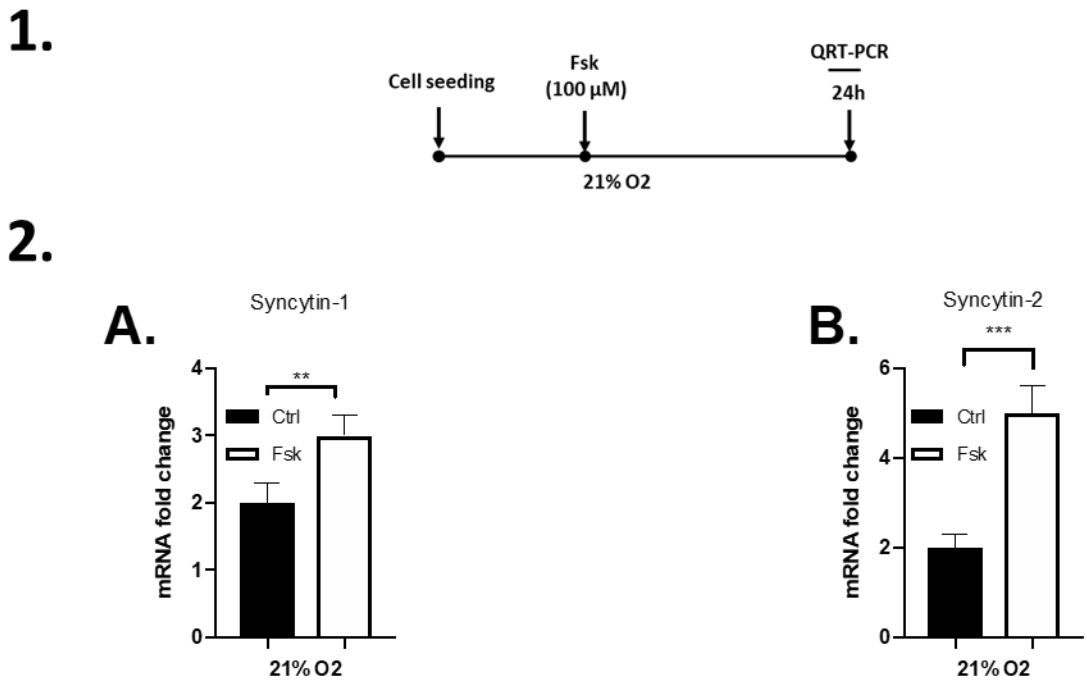


Figure 95

*CAMP/PKA signalling pathway increases syncytin 1 and 2 MRNA expression in BEWO cells. 1. Experiment protocol; where after 80% confluency, BEWO cells are treated with CAMP agonist for 24h. 2. QRT PCR analysis showed that the expression of (A) syncytin 1 and (B) syncytin 2 mRNA was significantly upregulated for 24h at 21% O2. ** .002 ,*** p=.0002; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.*

6.1.22 The effect of hypoxia on the expression of fusogenic genes in syncytialised bewo cells

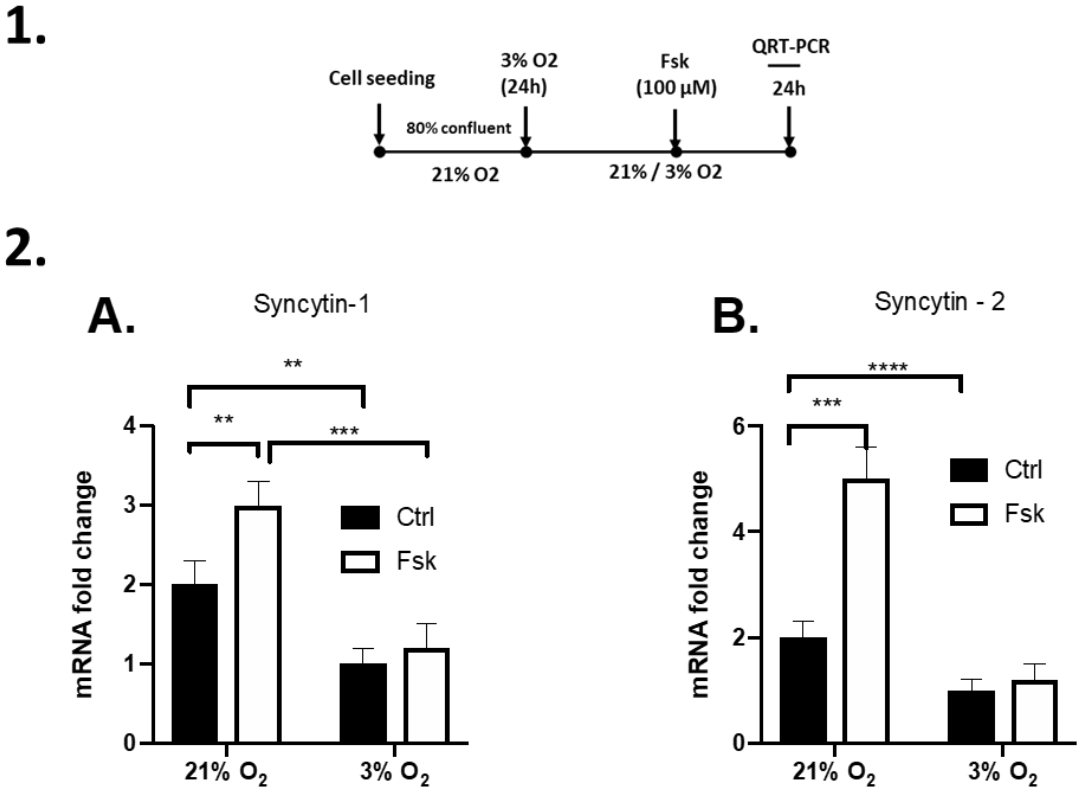


Figure 96

*Low oxygen tension impairs syncytin 1 and 2 gene expression induced by CAMP signalling pathway in BEWO cells. 1. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were treated with 100um forskolin for 24h. 2. QRT PCR analysis showed that low oxygen tension abolished upregulation of (A) syncytin 1 and (B) syncytin 2 induced by CAMP agonist. p= ** .002 *** .0002 ****< .0001; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using using two – way ANOVA with Tukeys multiple comparison test.*

6.1.23 Phospho CREB subcellular distribution induced by forskolin in BEWO cells : visualization by confocal microscopy

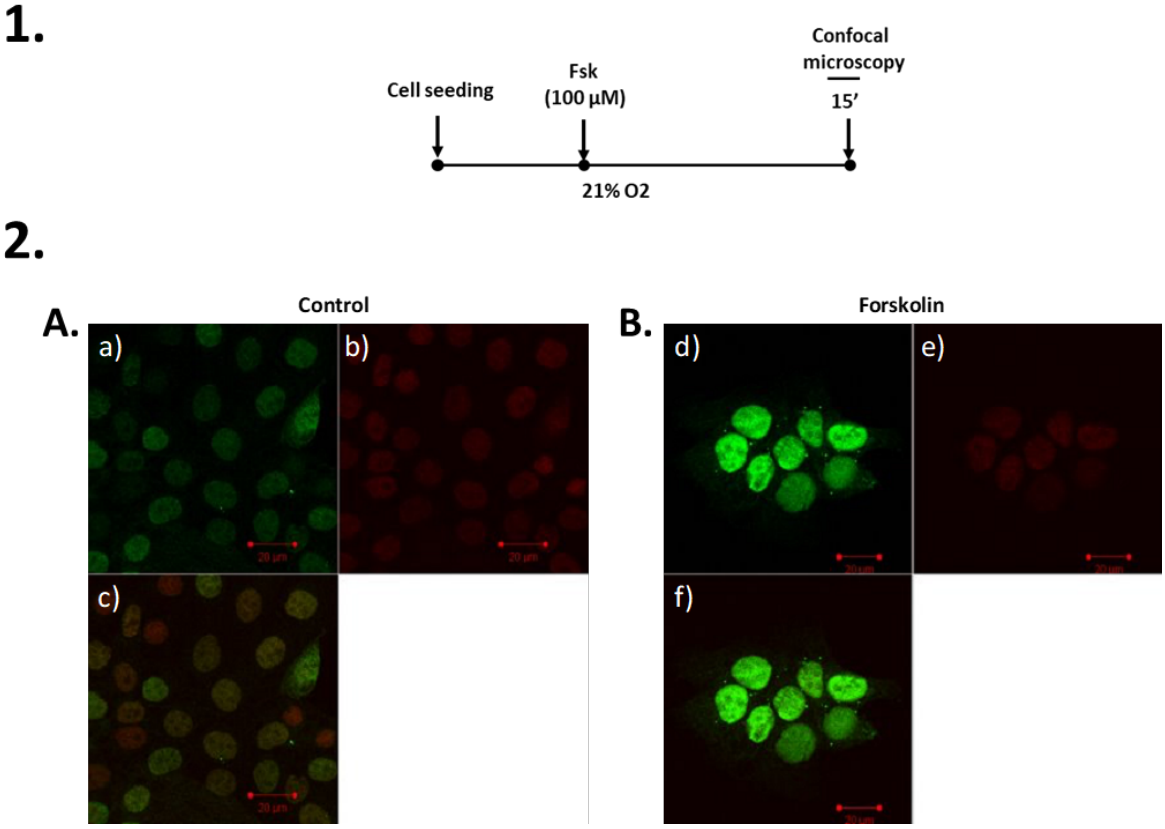


Figure 97

Forskolin induces BEWO cell fusion. Representative confocal microscopic images of sorted unfused BEWO cells and fused BEWO cells. 1.Experiment protocol; where after 80% confluency, cells were incubated with 100 µM forskolin for 15' and phospho –CREB Ser133 subcellular distribution in BEWO cells were detected using confocal microscopy. Phospho CREB (green) and CREB (red) distribution was monitored over 15' by indirect immunofluorescence using specific primary antibodies and FITC mouse 488 and Rabbit 563 secondary antibodies. Cell nuclei were stained with the DNA specific dye DAPI (blue) (data not shown). 2.(B) Confocal microscopy confirmed BEWO cells were mainly composed of fused cells characterised by a multinucleated syncytium. (A) In contrast, in absence of forskolin, the cell population contained mono-nucleated syncytium that displayed no change in cell size. Scale bar,20 µM; n=3.

6.1.24 The effect of hypoxia on the release of B-HCG, Progesterone and Cortisol in BEWO cells

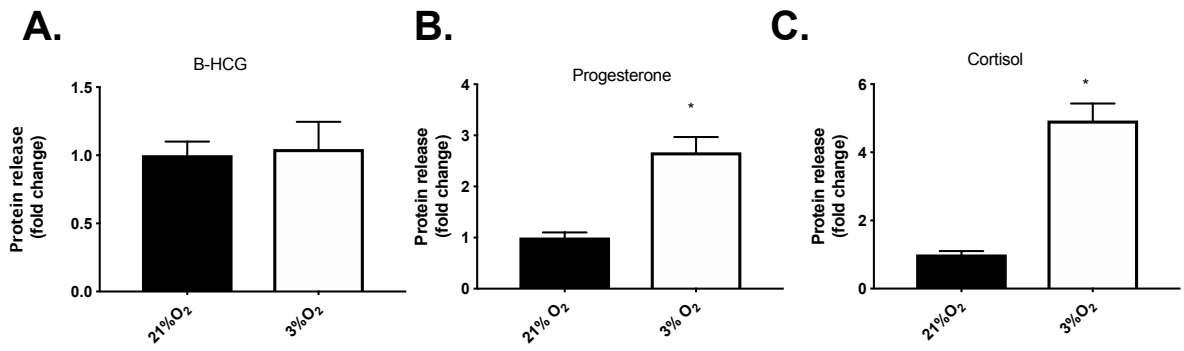


Figure 98

*Hypoxia increases progesterone and cortisol release, but not B-HCG release in BEWO cells. ECLIA analysis showed that the release of (B) progesterone and (C) cortisol but not (A) B-HCG was significantly increased at 3% O2 at 24h. *p= 0.033; n=3. Data expressed as fold change of protein release, relative to control 24h, after normalising against IU/mg protein. Data were analysed using a two-tailed unpaired t-test.*

6.1.25 The secretion of B-HCG, Progesterone, Estradiol and Cortisol in culture medium of BEWO cells in response to CAMP agonist

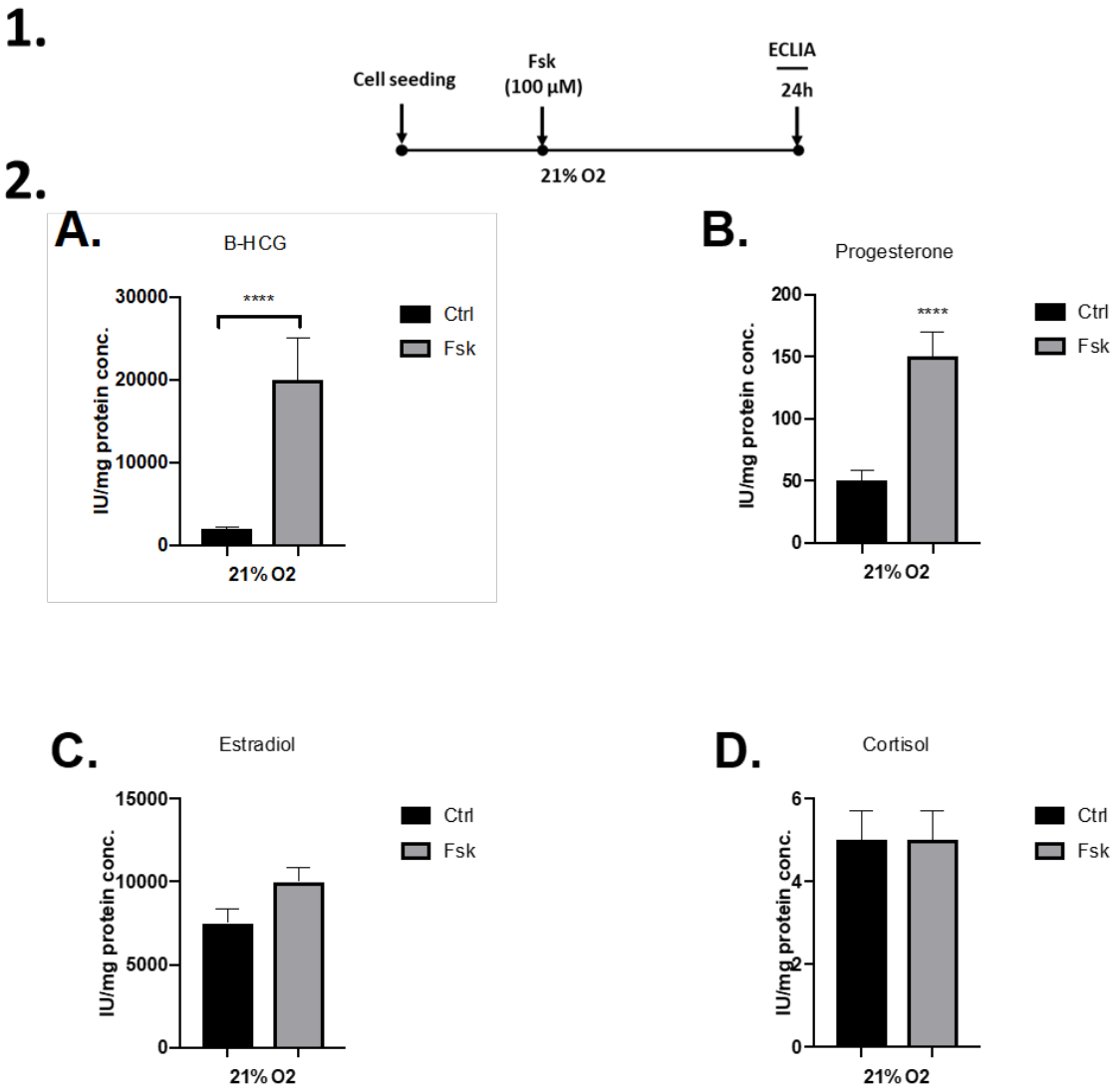


Figure 99

*CAMP/PKA signalling pathway increases protein release of B-HCG, progesterone but not estradiol and cortisol in BEWO cells. 1. Experiment protocol; where after 80% confluency, BEWO cells are treated with CAMP agonist for 24h. 2. MSD ECLIA assay showed that the release of (A) B-HCG and (B) progesterone, but not (C) estradiol and (D) cortisol was significantly increase for 24h at 21% O2. ****p< .0001; n=3. Data expressed as IU/mg protein concentration, relative to control 24h. Data were analysed using a two-tailed unpaired t-test.*

6.1.26 The effect of hypoxia on the endocrine capacity and biochemical differentiation in syncytialised BEWO cells

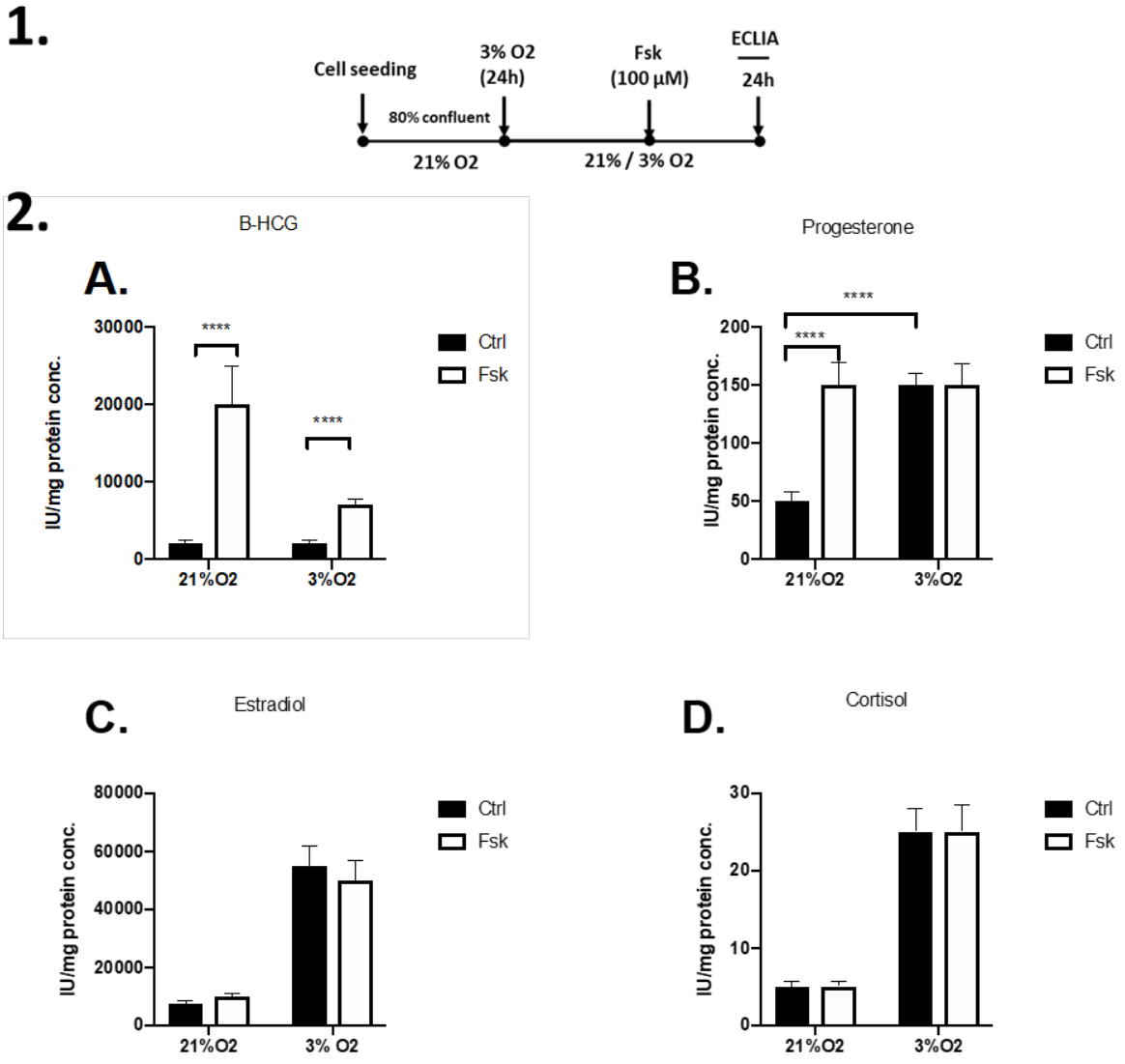


Figure 100

*Low oxygen tension impairs the enhanced release of B HCG and progesterone induced by cAMP signalling pathway in BEWO cells. 1. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were treated with 100um forskolin for 24h. 2. ECLIA analysis showed that low oxygen tension impaired enhanced release of B HCG and abolished progesterone release induced by CAMP agonist. Estradiol and cortisol release remained unaffected by low oxygen tension. ****p< .0001; n=3. Data expressed as IU/mg protein concentration, relative to control 24h. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

To determine the effect of the oxygen tension on the cell fusion, BeWo cells were cultured in either 21% or 3% oxygen in the presence or absence of forskolin and then the mrna expression of syncytin 1 and 2 were measured. Forskolin, a small lipophilic molecule easily to be absorbed and distributed, is a well-known adenylate cyclase activator and a widely reported differentiating agent in various tumors, including glioma cells [36,37] as well as in BEWO cells.

Syncytin 1 and 2 mrna expression, the measure of Cell fusion, were stimulated by the presence of forskolin at 24 h at 21% oxygen, which is further abolished at 3% O2. An associated increase in forskolin-induced hCG secretion by BeWo cells, a marker of syncytialisation, was more marked in 21% oxygen (20000 IU/mg protein conc.) compared to 3% oxygen(5000 IU/mg protein conc.). This was in accordance with https://www.sciencedirect.com/science/article/pii/S0925443903000437?via%3Dihub. This was further associated with an increase in progesterone release. The basal levels of estradiol (50000 IU/mg protein), progesterone (140 IU/mg protein) and cortisol (25 IU/mg protein conc.) in BEWO cells were increased at 3% O2. Hypoxia abolishes the increase in B-HCG (5000 IU/mg protein conc.) and progesterone release in BEWO syncytialised cells. No effect on estradiol and cortisol release was seen. Fusion was assessed by co-staining nuclei with phospho and total creb. Confocal microscopy stained with Pcreb Ser133 for 15' at 21% O2 further confirmed BEWO cells were mainly composed of fused cells characterised by a multinucleated syncytium. Cell fusion, as defined by the presence of multinucleated cells, was seldom seen in control cells, but multinucleated cells containing more than four nuclei could always be seen in cell cultures treated with either 100 µM forskolin 24 h. This indicates that forskolin was effective in promoting cytotrophoblast cell differentiation. It was necessary to further determine the effect of crh on trophoblast fusion and the effect of both crh and forskolin under altered oxygen tension.

6.1.27 The effect of CRH on the expression of fusogenic genes in syncytialised bewo cells

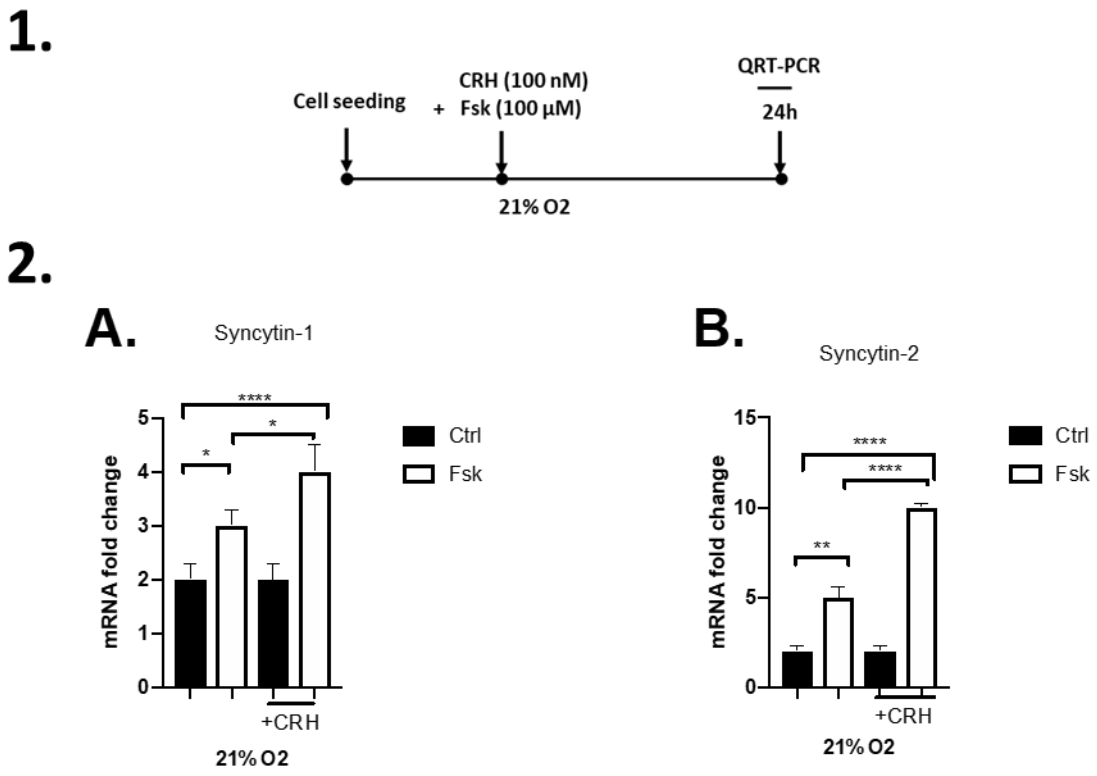


Figure 101

*CRH potentiates the actions of CAMP/PKA signalling pathway on the expression of syncytin 1 and 2 MRNA in BEWO cells. 1. Experiment protocol; where after 80% confluency, BEWO cells are treated with 100nm CRH and 100um forskolin for 24h at 21% O2. 2. QRT PCR analysis showed that CRH potentiated the actions of forskolin on syncytin 1 and 2 in BEWO cells at 24h. CRH alone had no effect on syncytin genes in BEWO cells at 24h. *p< 0.033, **p< .002, ****p< .0001; values are mean +/S.D.; n=3. Data expressed as fold change of gene expression, relative to control; at 24h, after normalising against B-actin. Data were analysed using a one way ANOVA with Tukeys multiple comparison test.*

6.1.28 The effect of CRH on the expression of fusogenic genes in syncytialised bewo cells under altered oxygen tension

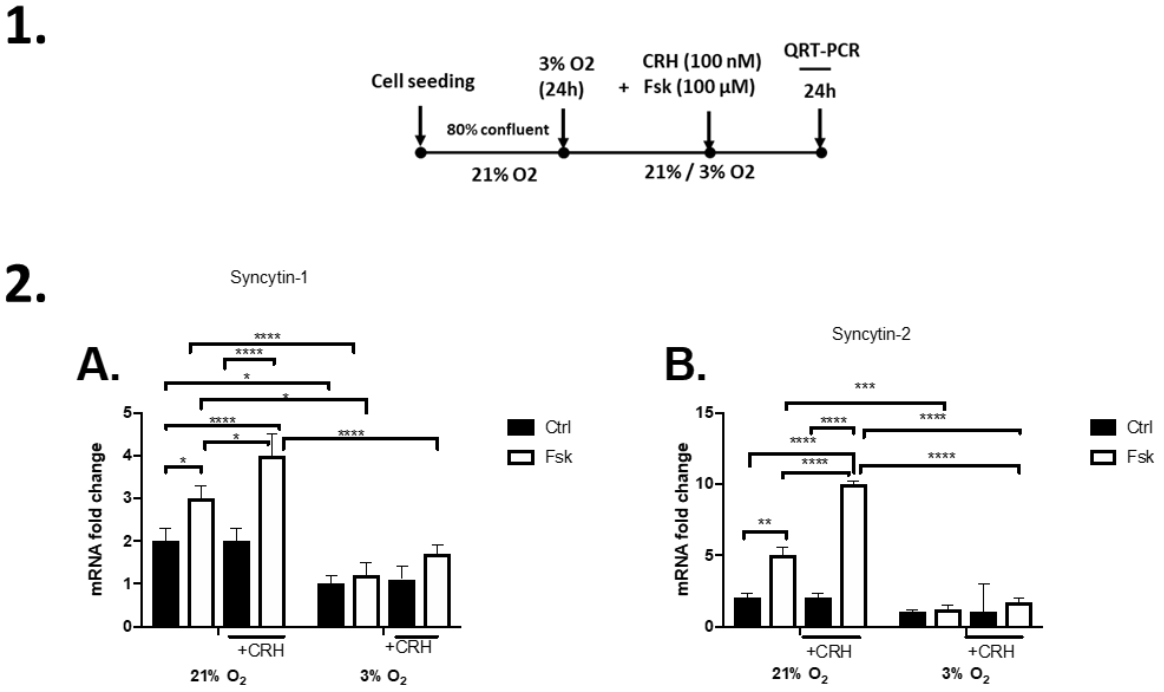


Figure 102

*Hypoxia abolishes the potentiation of syncytin expression by CRH in BEWO syncytialised cells. 1. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were treated with 100nm CRH and 100um forskolin for 24h. 2. QRT PCR analysis showed that under low oxygen tension, CRH was unable to potentiate the actions of forskolin on the MRNA expression of syncytin 1 and 2 in BEWO cells at 24h. CRH had no effect on syncytin genes expression under both high and low oxygen tensions. * p<0.033, ** .002, *** .0002 , ****p< .0001; values are mean +/S.D.; n=3. Data expressed as fold change of gene expression, relative to control at 24h, after normalising against B-actin. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

6.1.29 The effect of CRH on the endocrine capacity and biochemical differentiation in syncytialised BEWO cells

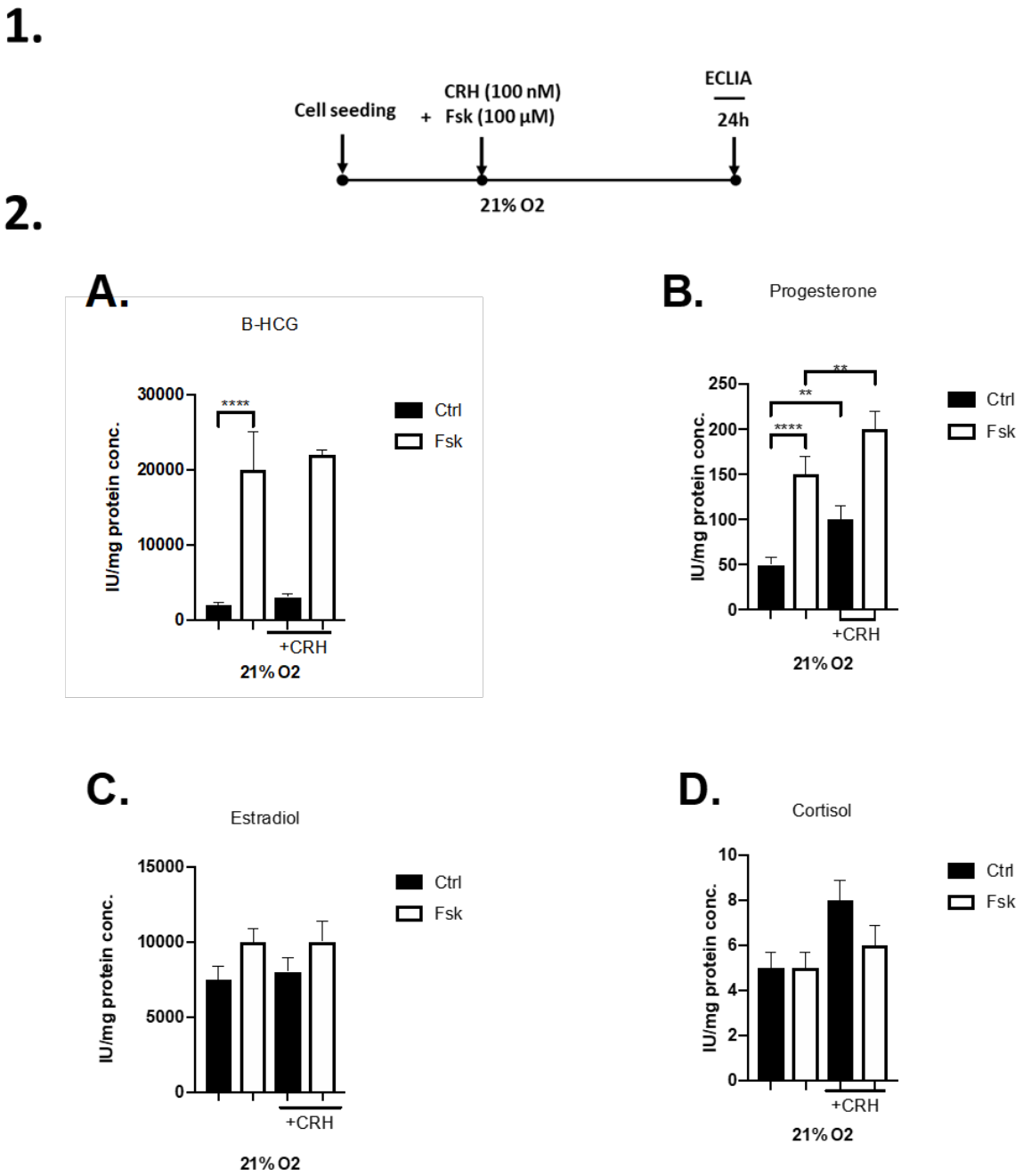


Figure 103

*CRH potentiates the actions of CAMP/PKA signalling pathway on progesterone release, but not B HCG, estradiol and cortisol in BEWO cells. 1. Experiment protocol; where after 80% confluency, BEWO cells are treated with 100nm CRH and 100um forskolin for 24h at 21% O2. 2. ECLIA analysis showed that CRH potentiated the actions of forskolin on progesterone protein release, but not B HCG, estradiol and cortisol release in BEWO cells at 24h. ** .002, ****p< .0001; values are mean +/S.D; n=3. Data expressed as IU/mg protein conc., relative to control 24h, after normalising against B-actin. Data were analysed using a one way ANOVA with Tukeys multiple comparison test.*

6.1.30 The effect of CRH on the endocrine capacity and biochemical differentiation in syncytialised BEWO cells under altered oxygen tension

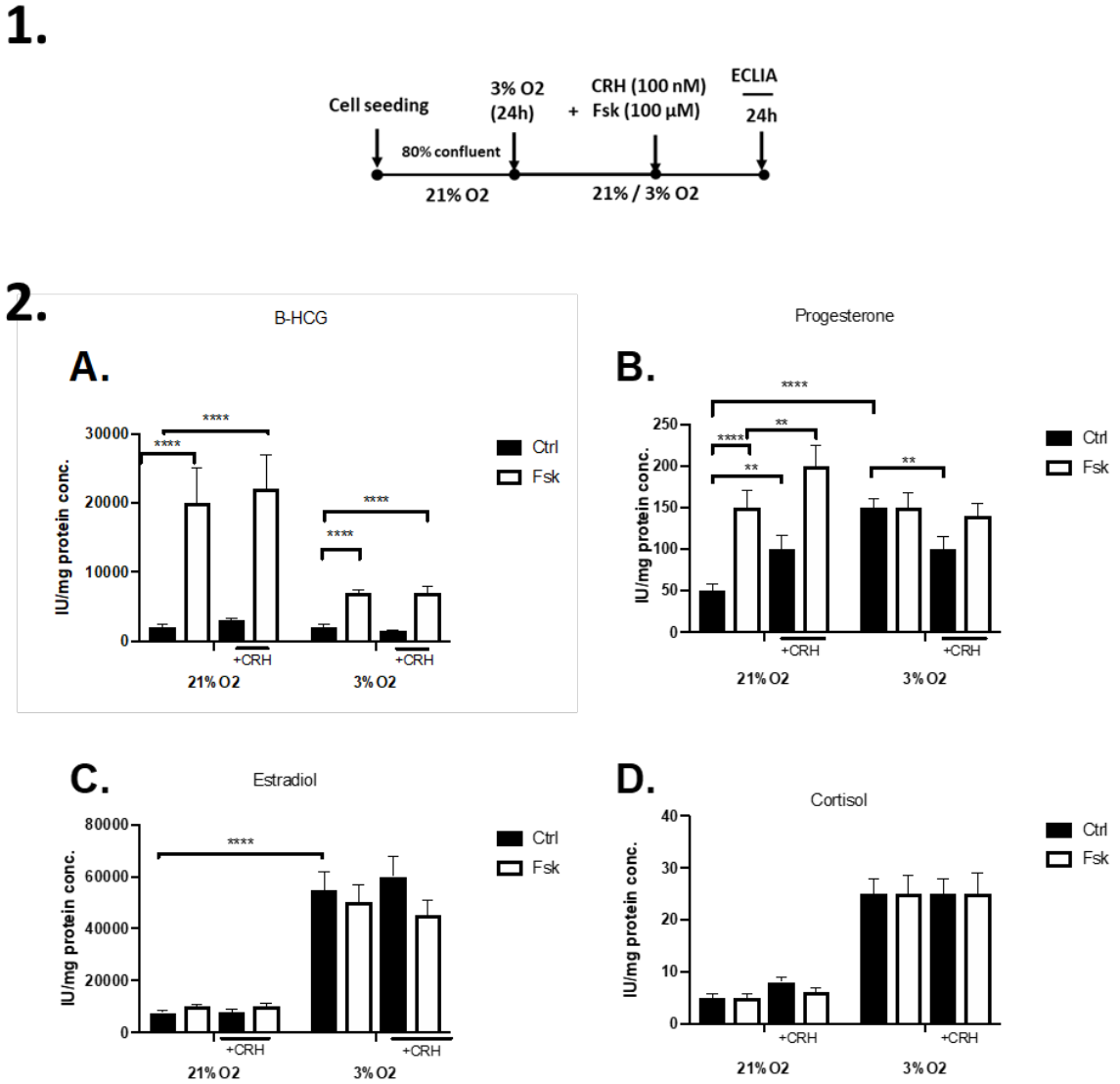


Figure 104

*under low oxygen tension, CRH reduces progesterone release in BEWO cells and CRH was unable to potentiate the actions of forskolin on progesterone at 24h. under low oxygen tension, CRH reduces progesterone release in unsyncytialised BEWO cells and the potentiation of progesterone in syncytialised BEWO cells. Hypoxia reversed the actions of CRH on progesterone release in BEWO cells. Hypoxia abolishes the potentiation of progesterone by CRH in BEWO syncytialised cells. Under low oxygen tension, CRH has no effect on B-HCG, estradiol and cortisol release in unsyncytialised and syncytialised BEWO cells. 1. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were treated with 100nm CRH and 100um forskolin for 24h. 2. ECLIA analysis showed that under low oxygen tension, CRH reduces progesterone release in BEWO cells and CRH was unable to potentiate the actions of forskolin on progesterone at 24h. ** .002, ****p< .0001; values are mean +/S.D; n=3. Data expressed as IU/mg protein concentration. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

As camp is a key component of the signalling pathway that promotes cytotrophoblast fusion, it was queried whether crh has any effect on advancing bewo cell differentiation under altered oxygen tension. The effects of forskolin and crh on the molecular markers of

cytotrophoblast differentiation, syncytin mrna expression and hormone secretion (such as B HCG, Estradiol and progesterone) were determined by qrt pcr and elisa assay respectively under both 21 and 3% O2.

CRH had no effect on syncytin 1 and 2 mRNA expression in BEWO cells at 21 % and 3% O2. In BEWO syncytialised cells, under low oxygen tension, the ability of CRH to potentiate the actions of forskolin on syncytin 1 and 2 mRNA expression in BEWO cells was lost. Forksolin and crh increases progesterone release at 21% o2. CRH potentiated the actions of forskolin on progesterone protein release, but not B HCG, estradiol and cortisol release in BEWO cells at 24h. cell differentiation is promoted by both forskolin and CRH. Hypoxia increases progesterone, estradiol and cortisol release in bewo cells.

Under low oxygen tension, CRH has no effect on B-HCG, estradiol and cortisol release in unsyncytialised and syncytialised BEWO cells. Hypoxia reversed the actions of CRH on progesterone release in BEWO cells. Hypoxia abolishes the potentiation of progesterone by CRH in BEWO syncytialised cells. Under low oxygen tension, CRH reduces progesterone release in unsyncytialised BEWO cells and the potentiation of progesterone in syncytialised BEWO cells. Forskolin increases b-hcg release and the effect is reduced under low oxygen tension. Crh potentiates the actions of forskolin on b-hcg release and hypoxia abolishes it. Crh has no effect on b-hcg release under high and low oxygen tension.

6.1.31 The effect of hypoxia on the expression of CRH receptors in BEWO cells

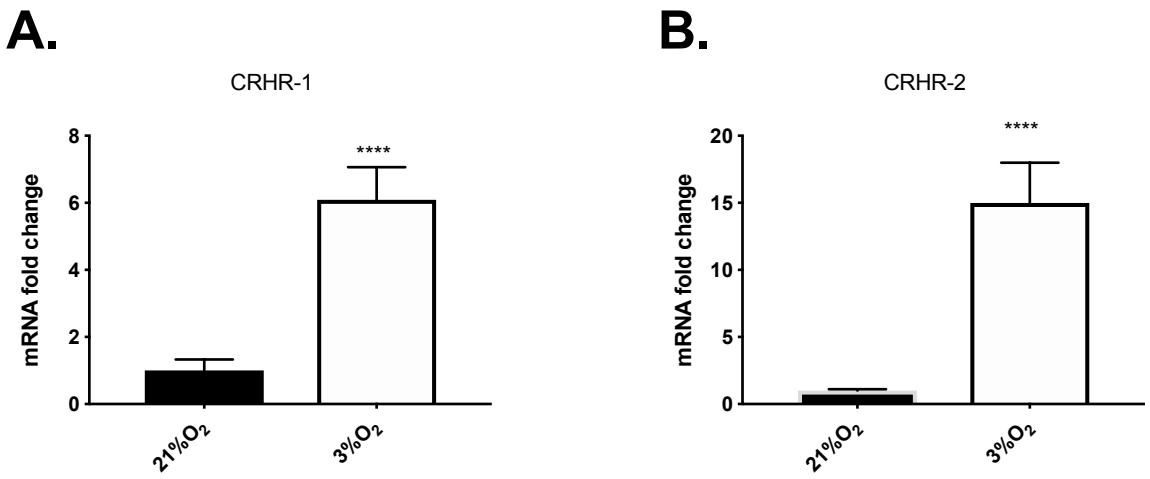


Figure 105

*Hypoxia upregulates the expression of CRHR-1 and 2 MRNA in BEWO cells. QRT PCR analysis showed that the expression of (A) CRHR 1 and (B) CRHR 2 mRNA was significantly upregulated for 24h at 3% O2. *** p=.0002; values are mean +/S.D ; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.*

6.1.32 The effect of CRH on the expression of CRHRs in syncytialised BEWO cells under altered oxygen tension

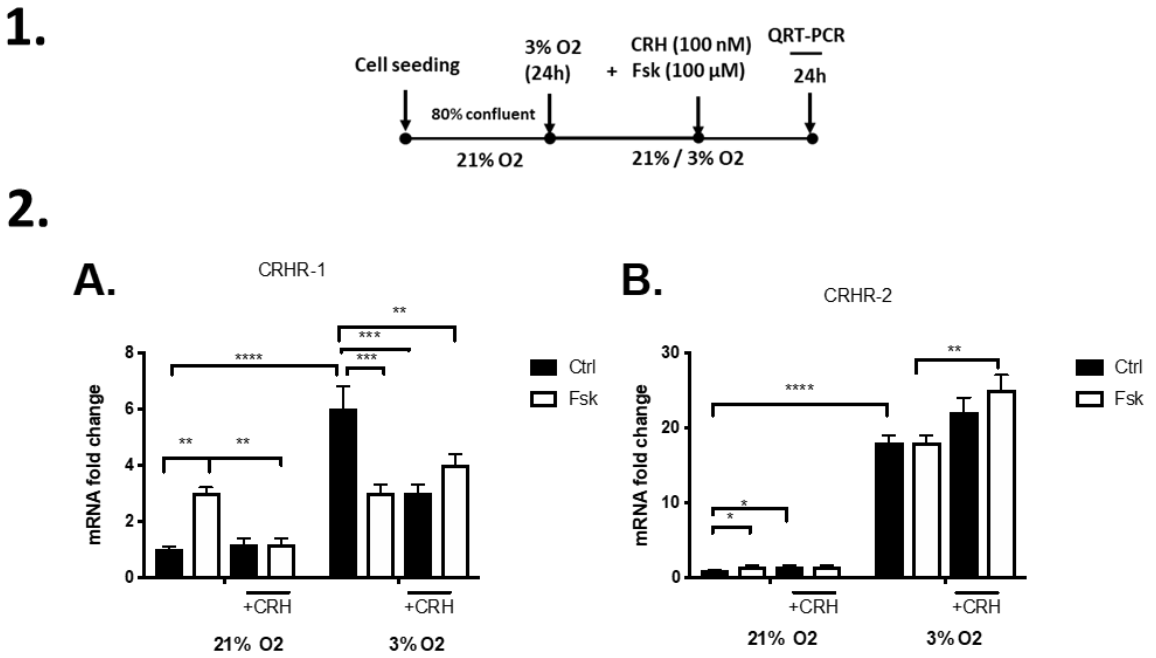


Figure 106

*Hypoxia upregulates CRHR1 and CRHR2 MRNA expression in BEWO cells. CRH potentiates CRHR2 expression in syncytialised BEWO cells. 1. Experimental protocol. 2. QRT PCR analysis showed that Forskolin increases (A) CRHR1 and (B) CRHR2 MRNA expression in BEWO cells. CRH has no effect on CRHR1 but increases CRHR2 mRNA expression in BEWO cells. CRH abolishes the effect of forskolin on MRNA expression of CRHR-1, but not CRHR2 in BEWO cells at 21% O2 at 24h. Hypoxia increases CRHR1 and CRHR2 expression in BEWO cells. Hypoxia reverses the effect of forskolin on CRHR1 expression in BEWO cells. Forskolin has no effect on CRHR2 MRNA expression in BEWO cells. Under low oxygen tension, CRH decreases CRHR1 but not CRHR2 MRNA expression in BEWO cells. Under low oxygen tension, CRH has no effect on CRHR1 expression but potentiates CRHR2 mrna expression in syncytialised BEWO cells. * 0.033, ** .002, *** .0002 , ****p< .0001; values are mean +/S.D; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-way ANOVA with Tukeys multiples comparison test.*

6.1.33 To determine the regulation of CRHRs by CRH and forskolin in BEWO cells under altered oxygen tension

Since crh receptors are crucial in mediating crh bioactivities, lack of potentiation of trophoblast fusion in response to crh under altered oxygen tension prompted us to examine

whether the regulation of crhr were being influenced by oxygen tension. Bewo cells were treated with 100nm crh for 24h and the levels of crhr1 and crhr2 mrna were quantified by qrt pcr. Crh upregulated the expression of crhr2 (2 fold) but not crhr1. This is against the study which showed upregulation of both crhrs in bewo cells by crh .https://rbej.biomedcentral.com/articles/10.1186/1477-7827-11-30#CR24 .Thus indicated that the CRH signalling pathway is intact and that the potential for a positive feed-forward pathway exists in BeWo cells.

It has been previously shown that 8-Br-cAMP increases CRH promoter activity in human primary placental cells [20]. The potential role of CRH on the cAMP-mediated effect on cell survival of BeWo cells under altered oxygen tension needs to be further explored. The effect of camp on crh promoter activity in bewo cells under altered oxygen tension as well needs to be further explored. The effect of forskolin on the expression of endogenous mrna encoding components of crh signalling pathway in bewo cells were examined. Bewo cells were treated with 100um forskolin and the level of crh receptors mrna was quantified by qrt pcr. Mrna levels for crh receptors were stimulated by forskolin. Following exposure to forskolin, the increase of crhr1 and crhr2 mrna was around 3 fold. This is similar to what has been reported earlier https://rbej.biomedcentral.com/articles/10.1186/1477-7827-11-30#CR5. In case mRNA is translated into protein, it may suggest that differentiated BeWo cells are more responsive to CRH cellular action. Hypoxia increases mrna expression of crhr1 (5.5 fold) and crhr2 (15 fold). Under altered oxygen tension, Forskolin reduces crhr1 mrna expression by 50%. The effect seen may be due to basal elevation of crhr1 mrna expression. Hypoxia abolishes the forskolin effect on crhr2 mrna expression.

Discussion

The differentiation of villous cytotrophoblast cells, the main cellular component of the placenta, is critical for a normal pregnancy as they mediate such steps as implantation, pregnancy hormone production, immune protection of the fetus, and delivery [1, 2]. It is known that upon in vitro stimulation with cAMP-inducing agents, such as forskolin, cytotrophoblasts undergo fusion into syncytiotrophoblasts. A syncytiotrophoblast is defined

as a giant cell with multiple nuclei sharing one cytoplasm, which expresses certain markers including syncytin 1 and human chorionic gonadotropin (hCG) [3, 4]. The syncytial mass, as assessed by the number of nuclei, increases exponentially across gestation [5]. Many factors are involved in the process of syncytialisation in the placenta, including cAMP-dependent protein kinase A, various protein tyrosine kinases, protein tyrosine phosphatases, syncytin-1, and GCM1 [4, 6]. While the list of players involved in cytotrophoblast differentiation is rapidly growing, the mechanisms remain far from clear. However, it is remarkable that little is known about the trophoblast cell fusion process (syncytiotrophoblast formation or syncytialisation). Mi et al. [1] suggested that syncytin, a protein encoded by an envelope gene of the recently identified human endogenous retrovirus-W (ERV-W) [2], may mediate placental cytotrophoblast fusion in vivo, and thus be important in human placental morphogenesis. In primary culture of isolated cytotrophoblast cells, the transcript levels of syncytin increase with the differentiation and fusion of cytotrophoblasts into syncytiotrophoblasts [3]. Recent experiments showed that under normoxic conditions (20% O2), relative mRNA abundance for syncytin is enhanced and for its receptor, amino acid transport system B0 (ASCT2), is suppressed in BeWo cells during syncytialisation by forskolin [6]. It has been reported that placental syncytin mRNA expression level is reduced in pregnancies complicated with pre-eclampsia [7] and its extreme form, hemolysis, elevated liver enzymes and low platelets syndrome [8]. The histological abnormalities of placentae in pregnancy associated with underperfusion and hypoxia are characterised by cytotrophoblast prominence and abnormalities in syncytiotrophoblast formation [10]. It also has been demonstrated that trophoblast cell fusion and differentiation are inhibited by hypoxia [11], [12], [13]. These observations have raised the possibility that low oxygen may, via regulation of syncytin gene expression and associated hormones such as B-HCG, suppress trophoblast cell fusion.

Corticotrophin releasing hormone (CRH), one of the hypothalamic “stress” peptides, plays a pivotal role in mammalian survival and adaptation responses involving the activation of the hypothalamic pituitary adrenal (HPA) axis. Besides its presence in the central nervous system, CRH is also expressed in the human placenta [7]. Placental CRH becomes detectable in maternal plasma around 16~20 weeks gestation, and increases exponentially as pregnancy progresses towards term. The level of placental CRH in maternal circulation has been linked to the length of gestation [8, 9, 10]. Placental CRH appears to target multiple feto-maternal

tissues, including the foetal adrenals, myometrial smooth muscle, placenta, placental vasculature and foetal membranes [11, 12, 13]. Through the various CRH receptor subtypes (CRHR1 and CRHR2) CRH plays diverse roles at different stages of pregnancy and labour. For example, CRH stimulates the foetal pituitary-adrenal axis, modulates placental vascular tone and endocrine function (especially prostaglandin generation), controls myometrial contractility ／ quiescence, and regulates trophoblast cell growth and invasion [10]. Nevertheless, there is little research literature available on a role for CRH in trophoblast cell differentiation.

Stimulation by 8-Br-cAMP of BeWo cells cultured in normal foetal calf serum resulted in higher levels of hCG and ERVW-1 [14]. The effect of 8-Br-cAMP on the expression of endogenous mRNA encoding components of the CRH signalling pathway was shown in BeWo cells. CRH promoter activity is increased by 8-Br-cAMP in human placental cells [15], while others have shown that CRH can act via CRH-receptors to increase cAMP production [16, 17]. Therefore, the potential roles of CRH on the differentiation of human trophoblast BeWo cells under altered oxygen tension were investigated.

It was found that under low oxygen tension, syncytin 1 and 2 mrna expression, B –HCG release is reduced in bewo syncytialised cells. It is not known if the effects can be reversed and needs to be further investigated. The progesterone release is abolished. Estradiol and cortisol release remains unaffected. This is associated with downregulation of CRHR1 and inability of CRHR2 upregulation. Further investigation on syncytin proteins and its receptors, amino acid transport system B are necessary to be established which are thought to be involved in trophoblast cell fusion (as well as the activity of amino acid transport through this system) in a cell model of syncytialisation (BeWo cells following forskolin treatment). Hypoxia increases progesterone, estradiol and cortisol release except B-HCG release. This is further associated with CRHR2 mRNA being more upregulated compared to CRHR1. These observations suggest that under conditions of low ambient oxygen, dysregulation of expression of syncytin and hormones may suppress the normal process of cell fusion necessary for syncytiotrophoblast formation and contributes to syncytiotrophoblast abnormalities characteristic of pre-eclampsia. This may perhaps be mediated via downregulation of crhr1 expression and loss of crhr2 upregulation.

CRH is known to activate the cAMP/PKA (protein kinase A) pathway via its receptors, CRHR1 and CRHR2, leading to phosphorylation of the cAMP response element binding protein (CREB1) [25]. As a transcription factor, CREB1 binds to a cAMP response element (CRE) sequence in the promoter of target genes, regulating the transcription of those genes [26, 27]. CRH has been studied as one of those target genes containing a CRE in the promoter, and the CRE has been shown to be crucial for CRH gene expression [28, 29, 30]. Furthermore, CRH and its receptors (CRHR1/R2) are expressed in placental tissues [9, 31] and CRH can bind to syncytiotrophoblast membranes [32]. So, the induction of CRH by cAMP hints that CRH may play a feedback role in cAMP mediated effects in placental trophoblasts.

Under high oxygen tension, CRH potentiates syncytins mRNA and progesterone release in syncytialised BEWO cells. CRHR1 and CRHR2 expression remains unaffected. The CRH effect on syncytins genes and progesterone release is lost under low oxygen tension. This is further associated with CRH having no effect on the downregulated CRHR1 expression in BEWO syncytialised cells but upregulates CRHR2 expression.

Since syncytialization was dysregulated if cells were cultured under altered oxygen tension, it led us to imply that CRH signal pathways may play a role in cAMP-mediated cell apoptosis and differentiation. The ability of CRH to regulate cell apoptosis in several cells of different origin has been reported [33, 34], and CRH has been shown to induce FASLG (Fas ligand) production and apoptosis in the rat pheochromocytoma cell line PC12 via activation of MAPK (p38 mitogen-activated protein kinase) [22]. It can thus be further proposed that although CRH is produced by differentiated trophoblasts it may also play a role in regulating trophoblast viability, thereby helping to keep the syncytiotrophoblast pool in renewal and this may be altered under low oxygen tension. Nevertheless, the existence of CRH receptors in BeWo cells suggests that CRH could be involved in another bioactivity, such as modulation of differentiation or syncytialisation of trophoblast cells. Consistent with previously published data, it was demonstrated that CRH can induce cell differentiation, as evidenced by the induction of ERVW-1 and by cell fusion. For trophoblast cells, the widely accepted marker of differentiation is a combination of a biochemical index (induction of hCG and ERVW-1 proteins) and a morphological index (formation of cell fusion), with the latter being more convincing. Recently, the reliability of hCG as a trophoblast differentiation marker has been

called into question, due to dissociation of the two differentiation indexes. Similar to what has been published, It was shown here additional evidence that biochemical and morphological differentiation can be dissociated, since CRH facilitates cell fusion without any induction of hCG secretion. Also, CRH stimulation induced ERVW-1 expression, without any induction of hCG secretion [35].

Similar dissociations have been reported in villous cytotrophoblast cells cultured in the absence of serum [36] and in JEG3 cells stimulated by forskolin, where an induction of hCG occurred without any evidence of cell fusion [21]. Recently, it was reported that the PKA inhibitor, H89, can reverse forskolin-induced BeWo cell fusion without altering forskolin-induced hCG secretion [37]. Therefore, hCG protein expression may not necessarily be linked to syncytial fusion. This study adds to the growing evidence that differentiation and fusion are related but distinct events [38]. However, the secretion of hCG can be enhanced by 8-Br-cAMP but not by CRH, suggesting that CRH and 8-Br-cAMP may regulate cell fusion through different signal pathways. Indeed, others have shown that charcoal scavenged hormones, such as estradiol, glucocorticoids and hCG, play roles in trophoblast differentiation at different stages of pregnancy [39].

That the expression of CRHR1 and CRHR2 can be up regulated by 8-Br-cAMP and also by CRH itself. There may exist a positive feed-forward mechanism in the CRH/CRHR/cAMP/PKA/CREB1 signal pathway, and that this occurs at several different levels. The positive communication existing with CRH and its receptors has the potential to magnify CRH bioactivities by, for example, promotion of trophoblast cell syncytialization. Since placental CRH expression in trophoblast cells begins when the cells differentiate toward syncytialization, the positive feed-forward and promotion of syncytialization by CRH could explain the exponential increase of CRH detected in maternal plasma as pregnancy progresses towards term, particularly as syncytial nuclear numbers increase exponentially across gestation [5]. This may provide the mechanism that links maturation of the placental structure to the timing of birth.

Table 45

	21%O2				3% O2			
	-Fsk		+Fsk		-Fsk		+Fsk	
	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH
Syncytin 1	NS	NS	↑	↑	↑	NS	NS	NS
Syncytin 2	NS	NS	↑	↑	↑	NS	NS	NS
B-HCG	NS	NS	↑	NS	NS	NS	↑	NS
Estradiol	NS	NS	NS	NS	NS	NS	NS	NS
Progesterone	NS	↑	↑	↑	↑	↓	NS	NS
Cortisol	NS	NS	NS	NS	↑	NS	NS	NS
CRHR-1	NS	NS	↑	↓	↑	↓	↓	NS
CRHR-2	NS	↑	↑	NS	↑	NS	NS	↑

6.1.34 The regulation of syncytialised BEWO cells under low and high oxygen tension by conditioned media obtained from HUVEC cells

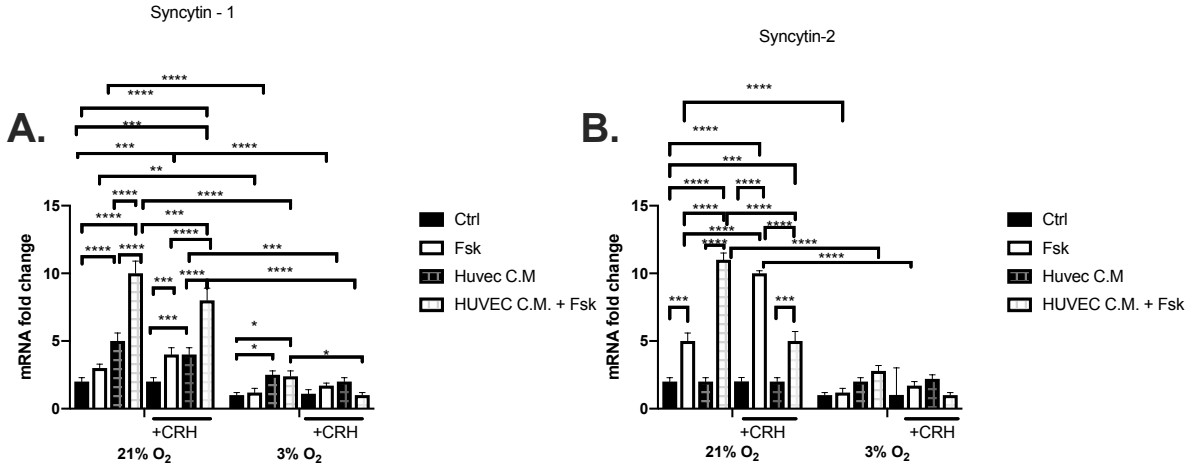


Figure 107

Under high oxygen tension, HUVEC conditioned media upregulates mRNA expression of syncytin 1 in unsyncytialised cells and syncytialised BEWO cells. Hypoxia reduces syncytin 1 mRNA expression in BEWO cells. Under low oxygen tension, HUVEC conditioned media upregulates mRNA expression of syncytin 1 in unsyncytialised BEWO cells. Under high oxygen tension, Syncytin 2 mRNA is upregulated in differentiated BEWO cells, which is further potentiated by HUVEC conditioned media. These effects are abolished in low oxygen tension. CRH has no effect on syncytin 1 and syncytin 2 mRNA expression in high and low oxygen tension. CRH potentiates the actions of syncytin 2 mRNA in differentiated BEWO cells under high oxygen tension. This is further lost in low oxygen tension. CRH potentiates the actions of HUVEC conditioned media on syncytin 1 in undifferentiated BEWO cells under high oxygen tension, which is lost under low oxygen tension. CRH potentiates the actions of HUVEC conditioned media on syncytin 1 in differentiated BEWO cells under high oxygen tension, which is reversed and reduces in hypoxia. CRH reduces the actions of HUVEC conditioned media on syncytin 2 in differentiated BEWO cells under high oxygen tension, which is lost in hypoxia.

Table 46

	Syncytin -1		Syncytin -2	
	21% O2	3% O2	21% O2	3% O2
Ctrl	NS	↓	NS	NS
Fsk	NS	NS	↑	NS
HUVEC C.M.	↑	↑	NS	NS
HUVEC C.M. + Fsk	↑	NS	↑	NS
CRH	NS	NS	NS	NS
CRH + Fsk	↑	NS	↑	NS

HUVEC C.M. + CRH	↑	**NS**	**NS**	**NS**
HUVEC C.M. + CRH + Fsk	↑	↓	↓	**NS**

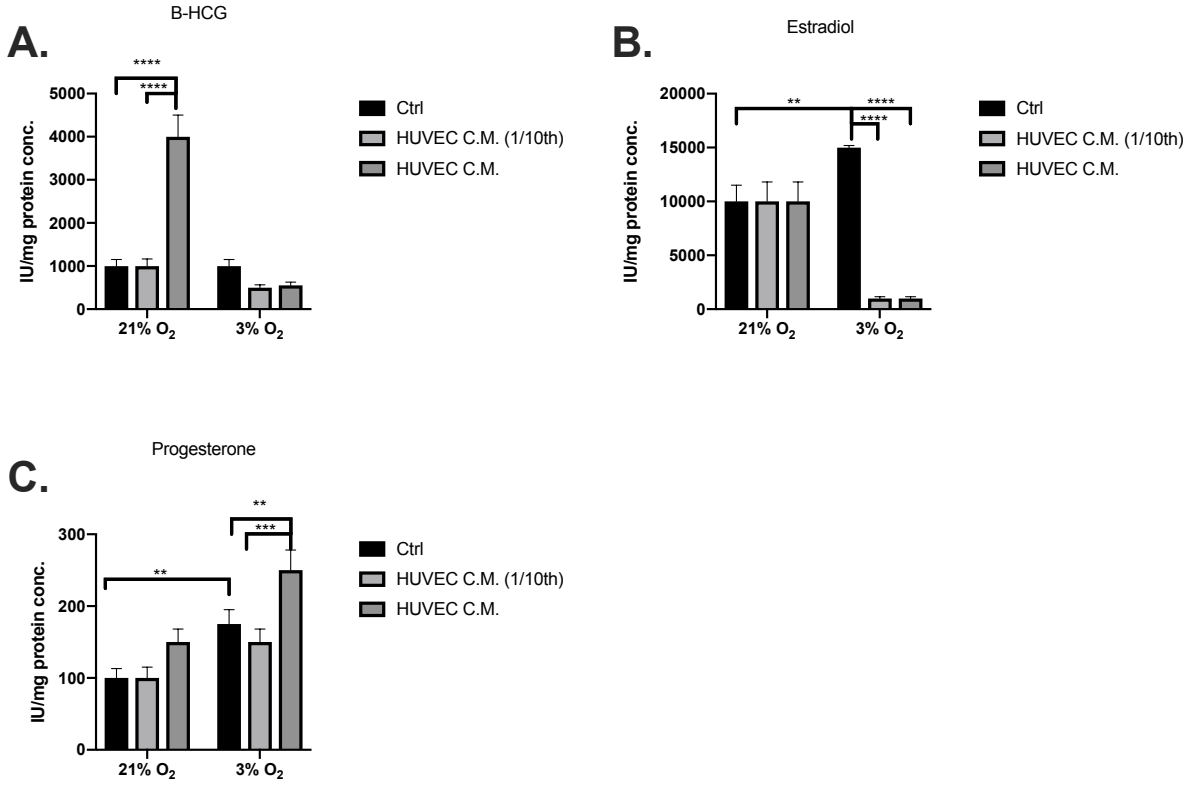


Figure 108

Conditioned media obtained from HUVECs releases maximum B-HCG protein release from BEWO cells under high oxygen tension at 24h, which is further lost under hypoxia. Hypoxia increases estradiol and progesterone release from BEWO cells under low oxygen tension. Hypoxia potentiates actions of HUVEC conditioned media on progesterone release .

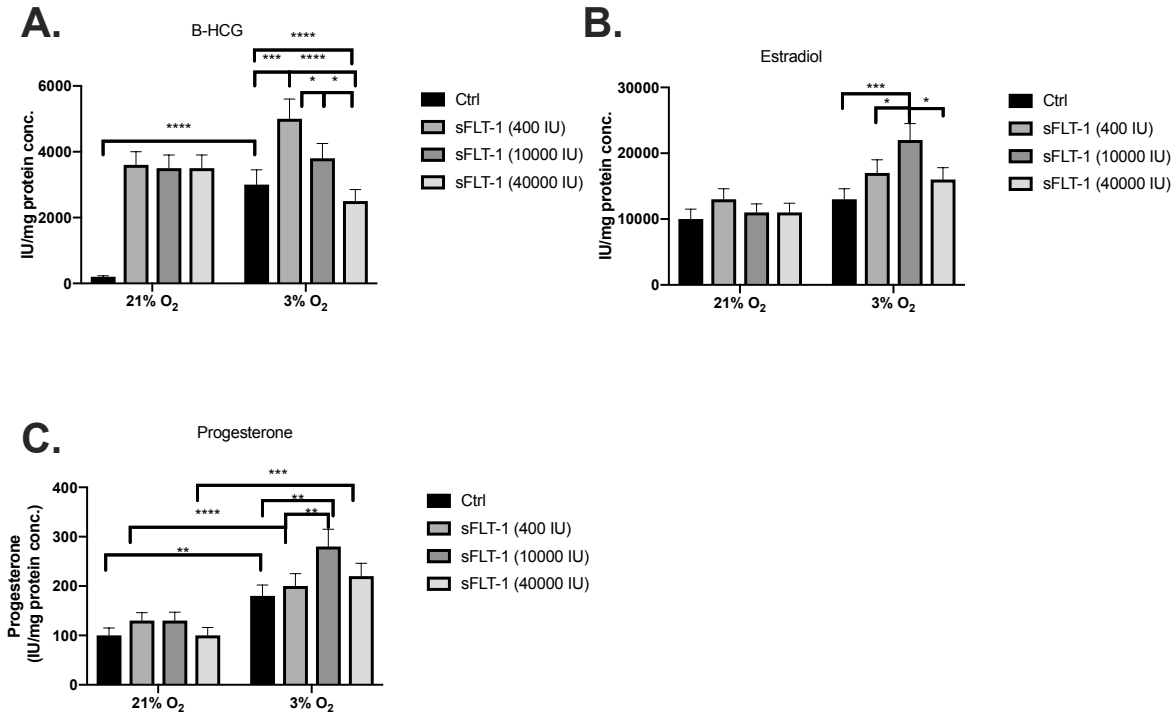


Figure 109

Maximum B HCG protein release is seen with all the 3 doses of sFLT-1 (400IU, 10K IU, 40K IU) under high oxygen tension at 24h in undifferentiated BEWO cells. Hypoxia increases protein release of B-HCG and progesterone. sFLT-1 (10K) causes maximum estradiol and progesterone release under low oxygen tension in undifferentiated BEWO cells.

Table 47

	B HCG		**Estradiol**		**Progesterone**	
	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2
Ctrl	NS	↑	NS	↑	NS	↑
1/10th HUVEC C.M.	NS	NS	NS	↓	NS	NS
HUVEC C .M.	↑	NS	NS	↓	NS	↑
sFLT-1 (400IU)	↑	↑	NS	NS	NS	NS
sFLT-1 (10K IU)	↑	↓	NS	↑	NS	↑
sFLT-1 (40K IU)	↑	↓	NS	NS	NS	NS

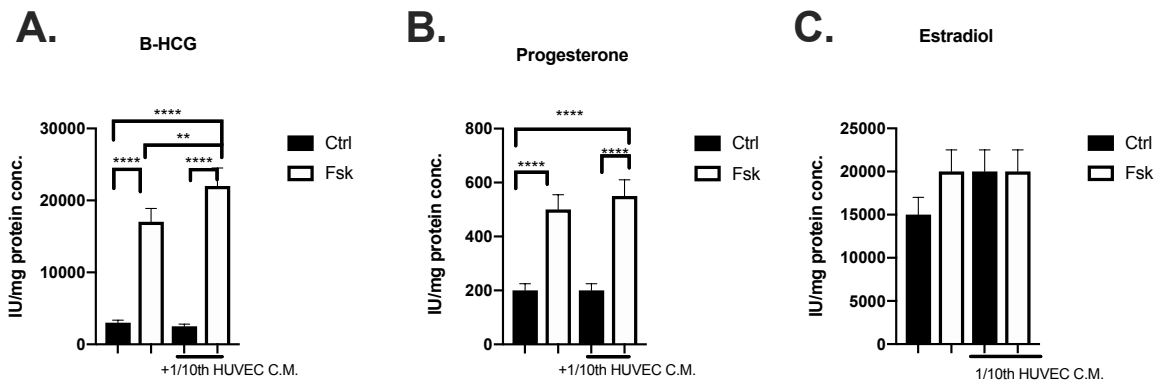


Figure 110

Forskolin increases B -HCG, progesterone and estradiol release under high oxygen tension at 24h in BEWO cells. 1/10th of the original dose of conditioned media obtained from HUVEC causes no change in B HCG, progesterone and estradiol release. 1/10th of the original dose of conditioned media obtained from HUVEC causes increased release of B-HCG but not progesterone and estradiol in differentiated BEWO cells.

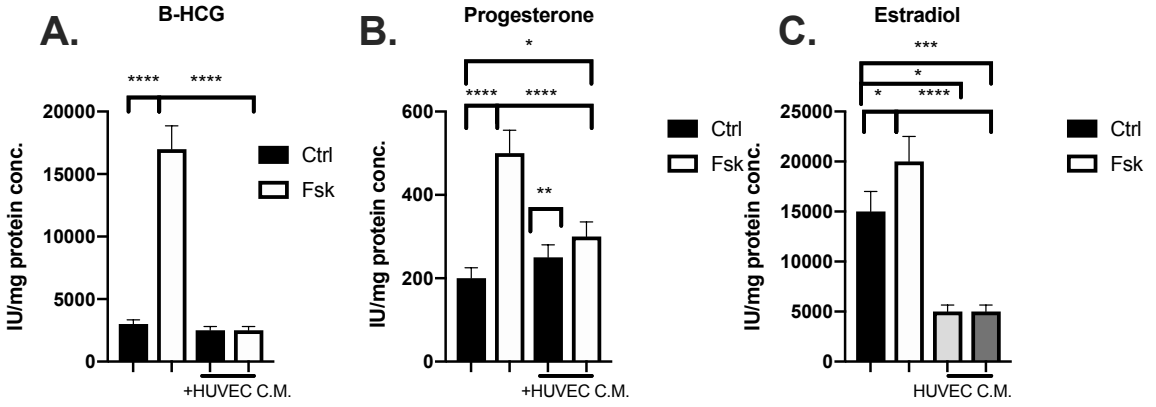


Figure 111

Forskolin increases the release of B-HCG , progesterone and estradiol. HUVEC conditioned media reduces estradiol release but has no effect on B-HCG, progesterone release in undifferentiated BEWO cells. HUVEC conditioned media reduces B-HCG, progesterone and estradiol release in differentiated BEWO cells.

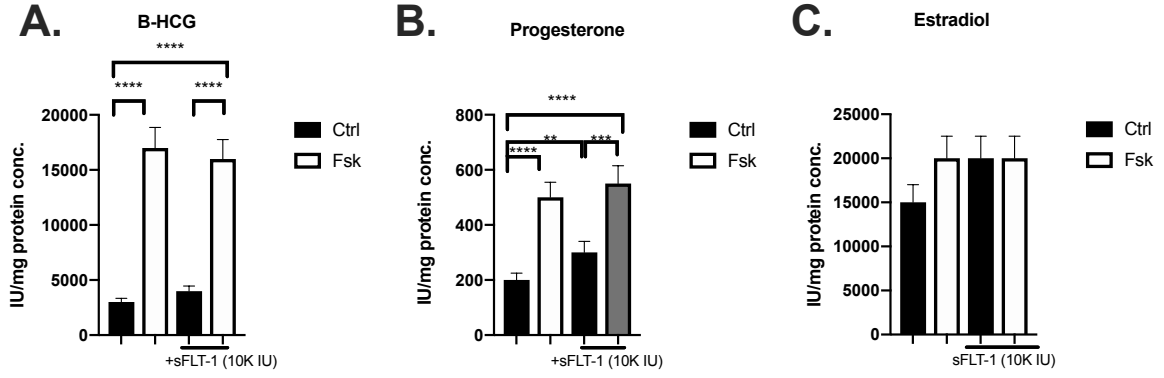


Figure 112

Forskolin increases B-HCG, progesterone and estradiol release in BEWO cells. sFLT-1 (10K IU) increases progesterone and estradiol but not B -HCG release in undifferentiated BEWO cells under high oxygen tension. sFLT-1 (10K IU) has no effect on B-HCG, progesterone and estradiol release in differentiated BEWO cells under high oxygen tension.

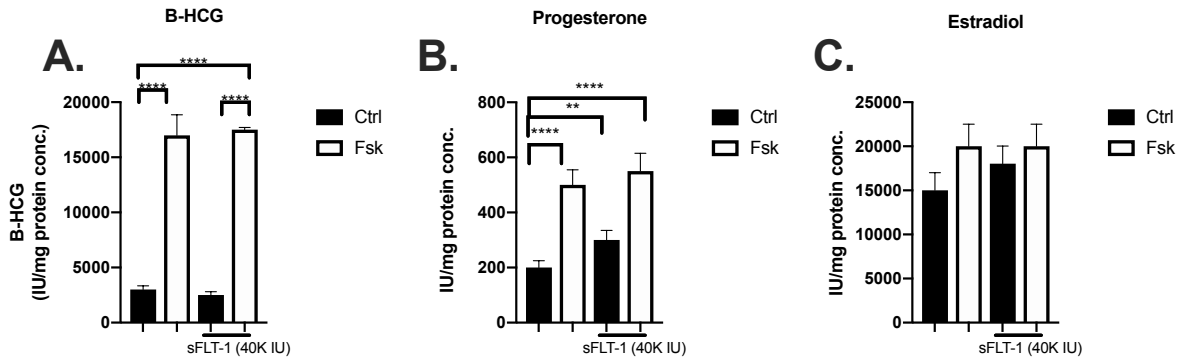


Figure 113

sFLT-1 (40K IU) increases progesterone but not B -HCG and estradiol release in undifferentiated BEWO cells under high oxygen tension. sFLT-1 (40K IU) has no effect on B-HCG, progesterone and estradiol release in differentiated BEWO cells under high oxygen tension.

Table 48

	B HCG	Estradiol	Progesterone
Ctrl	NS	NS	NS
Fsk	↑	↑	↑
1/10th HUVEC C.M.	NS	NS	NS
1/10th HUVEC C.M. + Fsk	↑	NS	NS
HUVEC C.M.	NS	↓	NS
HUVEC C.M. + Fsk	↓	↓	↓
SFLT-1 (10K IU)	NS	NS	NS
SFLT-1 (10K IU) + Fsk	NS	NS	NS
SFLT-1 (40K IU)	NS	NS	NS
SFLT-1 (40K IU) + Fsk	NS	NS	NS

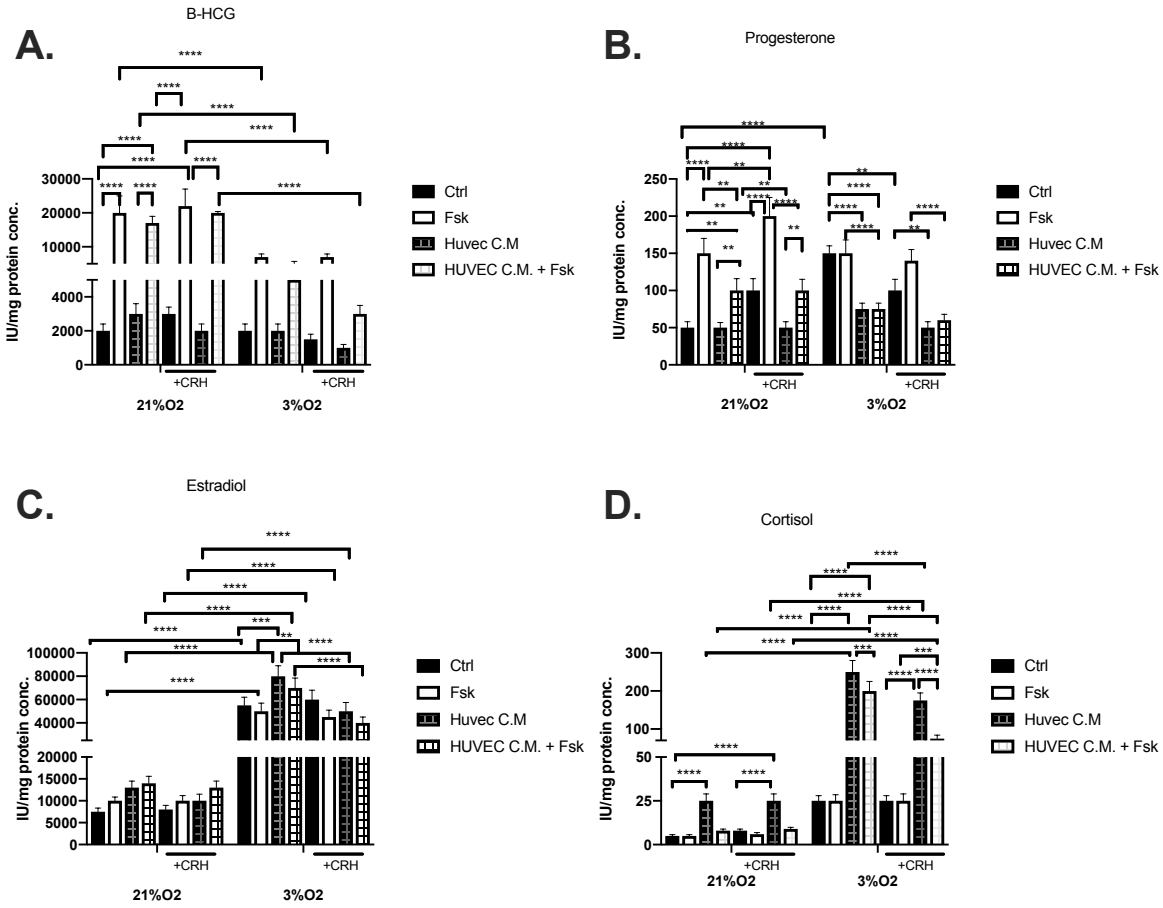


Figure 114

CRH increases progesterone release but has no effect on estradiol release under high oxygen tension in undifferentiated and differentiated BEWO cells. CRH decreases progesterone release under low oxygen tension in undifferentiated bewo cells. CRH has no effect on cortisol release under high oxygen tension in undifferentiated and differentiated BEWO cells. crh reduces HUVEC C.M. mediated cortisol release in undifferentiated and differentiated BEWO cells under high and low oxygen tension. CRH has no effect on B HCG release in undifferentiated and differentiated BEWO cells under high and low oxygen tension. CRH has no effect on HUVEC C.M. mediated B HCG release in undifferentiated and differentiated BEWO cells under high and low oxygen tension.

Table 49

	B HCG		Progesterone		Estradiol		Cortisol	
	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2
Ctrl	NS	NS	NS	↑	NS	NS	NS	NS
Fsk	↑	↓	↑	NS	NS	NS	NS	NS
HUVEC C.M.	NS	NS	NS	↓	NS	↑	↑	↑

HUVEC C.M. + Fsk	NS	NS	↓	↓	NS	↑	NS	↓
CRH	NS	NS	↑	↓	NS	NS	NS	NS
CRH + Fsk	↑	NS	↑	NS	NS	NS	NS	NS
HUVEC C.M. + CRH	↓	NS	NS	↓	NS	↓	NS	↑
HUVEC C.M. + CRH + Fsk	NS	↓	NS	NS	NS	↓	NS	↓

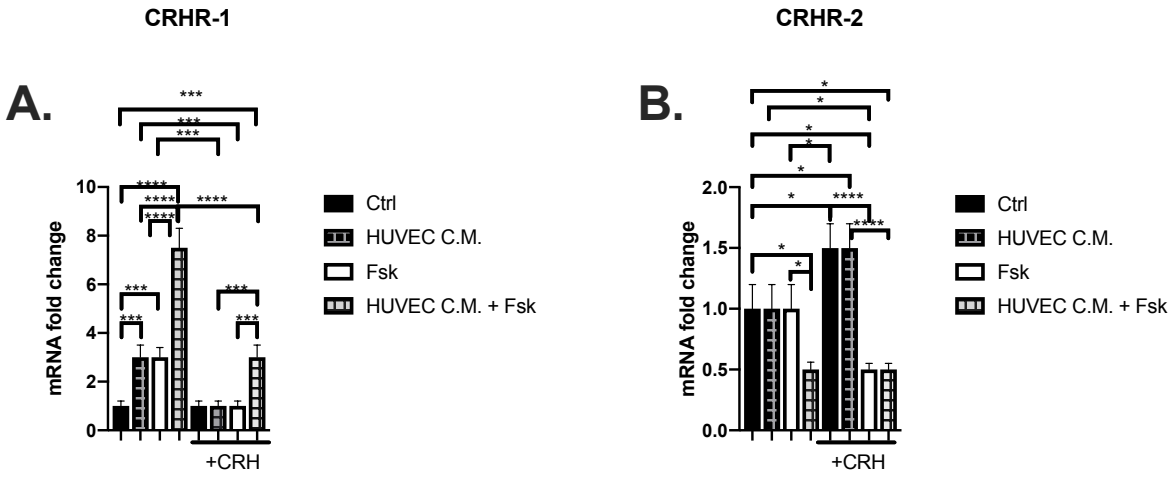


Figure 115

Conditioned media obtained from HUVECs upregulates CRHR-1 mRNA but not CRHR2 mRNA expression under high oxygen tension at 24h. Forskolin increases CRHR1 mRNA but not CRHR2 mRNA. Huvec conditioned media increases CRHR1 mRNA expression in undifferentiated and differentiated bewo cells. Huvec conditioned media does not affect CRHR2 MRNA in undifferentiated bewo cells but reduces CRHR2 mRNA expression in differentiated bewo cells.CRH increases CRHR2 mRNA but does not affect CRHR1 mRNA. Forksolin does not upregulate CRHR1mRNA in presence of CRH. HUVEC conditioned media does not upregulate CRHR1mRNA in presence of CRH. CRH reduces the ability of HUVEC conditioned media to upregulate CRHR1mRNA in differentiated BEWO cells. HUVEC conditioned media increases CRHR2mRNA in presence of CRH in undifferentiated BEWO cells. CRH decreases CRHR2mRNA in differentiated BEWO cells.The ability of HUVEC conditioned media to reduce CRHR2 mRNA expression in differentiated BEWO cells remain unaffected in presence of CRH.

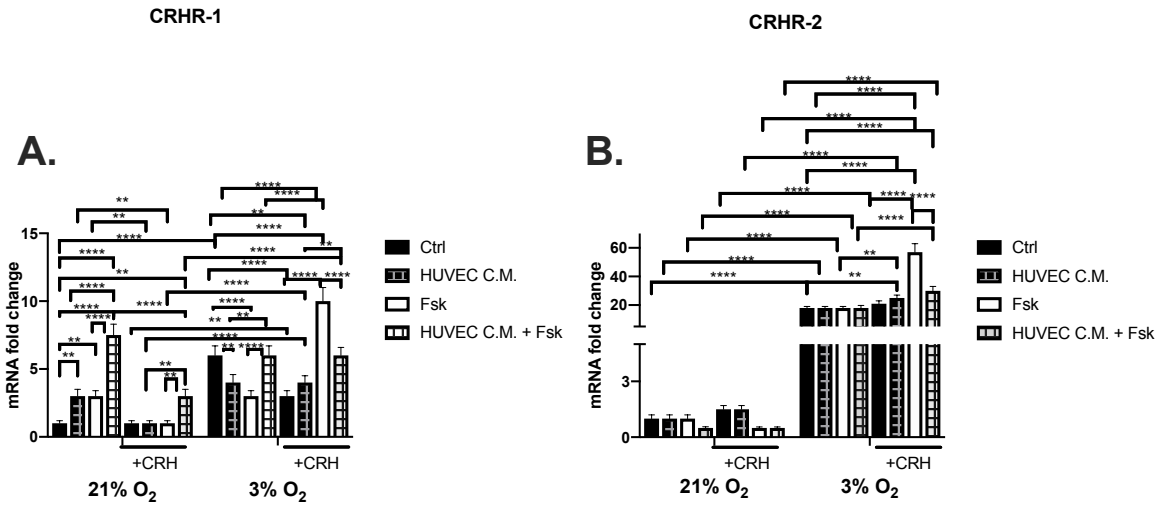


Figure 116

HUVEC conditioned media increases CRHR1 mRNA expression in undifferentiated and differentiated BEWO cells. Forskolin increases CRHR1 mRNA expression. CRH has no effect on CRHR1 mRNA expression in undifferentiated BEWO cells. CRH prevents the ability of forskolin to increase CRHR1 mRNA. CRH prevents the ability of HUVEC conditioned media to increase CRHR1 mRNA in undifferentiated BEWO cells. CRH reduces the ability of HUVEC conditioned media to increase CRHR1 mRNA in differentiated BEWO cells.

Hypoxia increase CRHR1 mRNA expression. HUVEC conditioned media reduces CRHR1 mRNA. Forskolin reduces CRHR1 mRNA. HUVEC conditioned media reverses the actions of forskolin on CRHR1 mRNA.CRH reduces CRHR1 mRNA in hypoxia.CRH does not affect the ability of HUVEC conditioned media to regulate CRHR1 mRNA.CRH reverses the actions of forskolin on CRHR1 mRNA. HUVEC conditioned media increases CRHR1 mRNA in differentiated BEWO cells and CRH has no effect on this.

HUVEC conditioned media does not affect CRHR2 mRNA expression under high oxygen tension at 24h. HUVEC conditioned media does not affect CRHR2 mRNA in undifferentiated BEWO cells but reduces CRHR2 mRNA expression in differentiated BEWO cells. CRH increases CRHR2mRNA but does not affect CRHR1 mRNA. HUVEC conditioned media increases CRHR2 in presence of CRH in undifferentiated BEWO cells. CRH decreases CRHR2 mRNA in differentiated BEWO cells. The ability of HUVEC conditioned media to reduce CRHR2 mRNA expression in differentiated BEWO cells remain unaffected in presence of CRH.

Hypoxia increases CRHR2 mRNA. HUVEC conditioned media increases CRHR2 mRNA in presence of CRH in hypoxia. Forskolin increases CRHR2 mRNA in presence of CRH in hypoxia.

Table 50

	CRHR-2	
	21% O2	3% O2
Ctrl	NS	↑
Fsk	NS	NS
HUVEC C.M.	NS	NS
HUVEC C.M. + Fsk	↓	NS
CRH	↑	NS

CRH + Fsk	↓	↑
HUVEC C.M. + CRH	↑	↑
HUVEC C.M. + CRH + Fsk	↓	↑

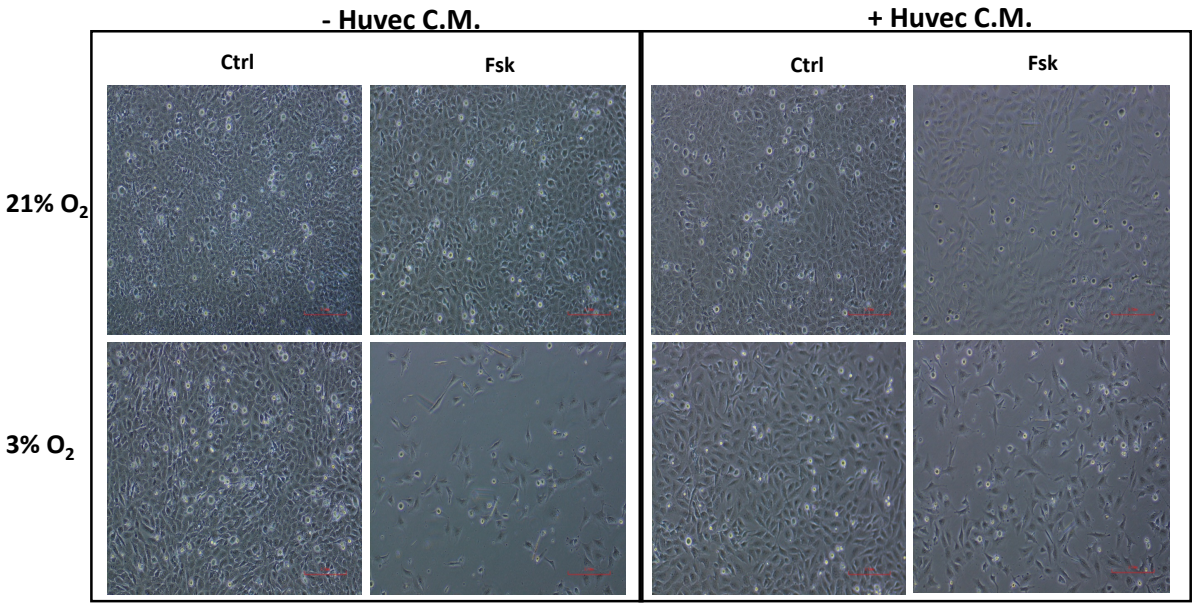
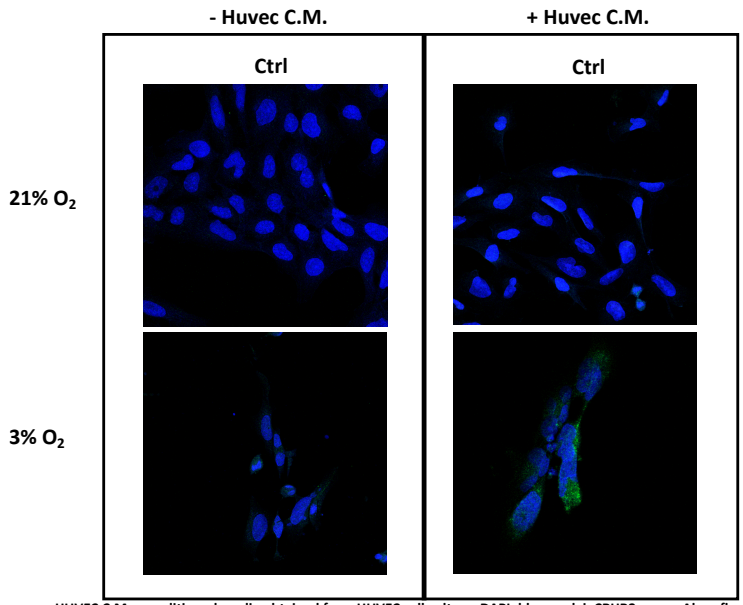


Figure 117

Forskolin reduces cellular number and induces apoptosis in BEWO cells under low oxygen tension at 24h. HUVEC conditioned media has no effect on undifferentiated and differentiated cells under high and low oxygen tension.



CRH: corticotrophin releasing hormone; HUVEC C.M. : conditioned media obtained from HUVEC cell culture; DAPI: blue nuclei; CRHR2: green Alexa fluor 488

Figure 118

No change in CRHR2 mRNA expression is seen in BEWO cells in low and high oxygen tension in presence or absence of HUVEC conditioned media.

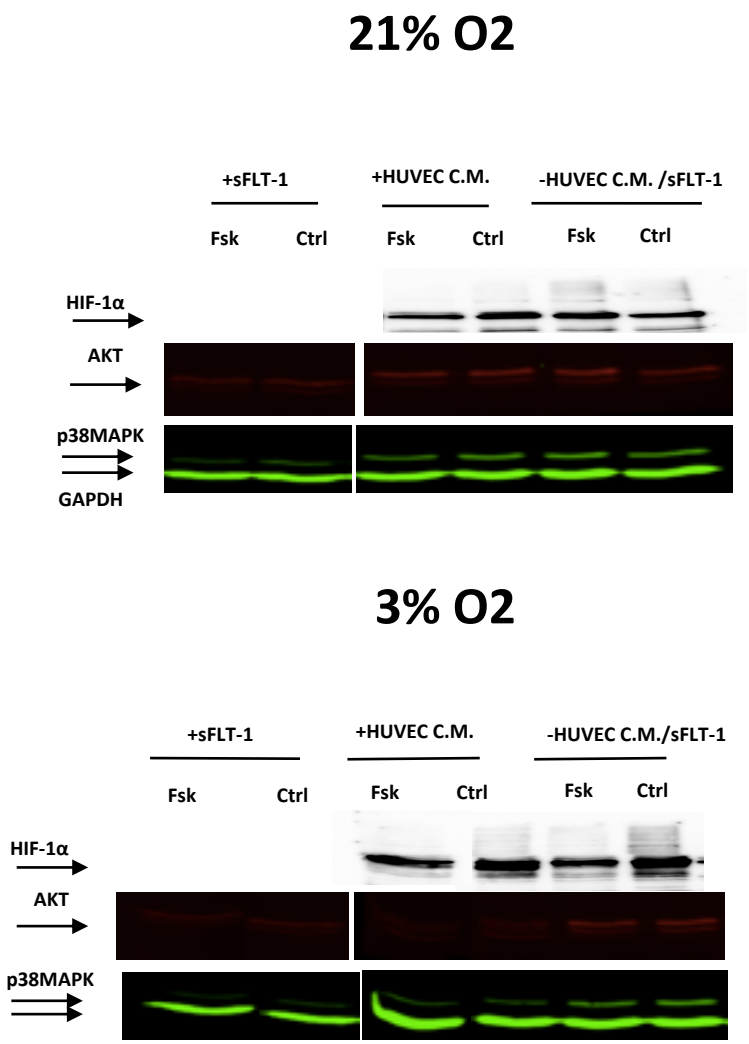


Figure 119

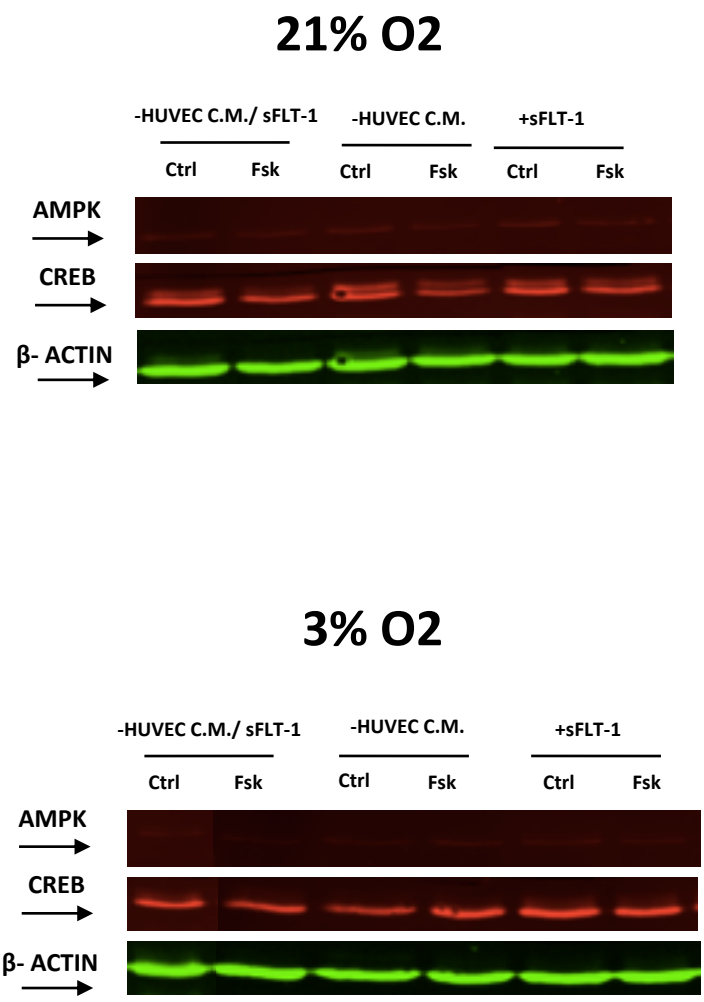

21% O2
-HUVEC C.M./ sFLT-1
-HUVEC C.M.
+sFLT-1
Ctrl
Fsk
Ctrl
Fsk
Ctrl
Fsk
AMPK
CREB
β- ACTIN
3% O2
-HUVEC C.M./ sFLT-1
-HUVEC C.M.
+sFLT-1
Ctrl
Fsk
Ctrl
Fsk
Ctrl
Fsk
AMPK
CREB
β- ACTIN

Figure 120

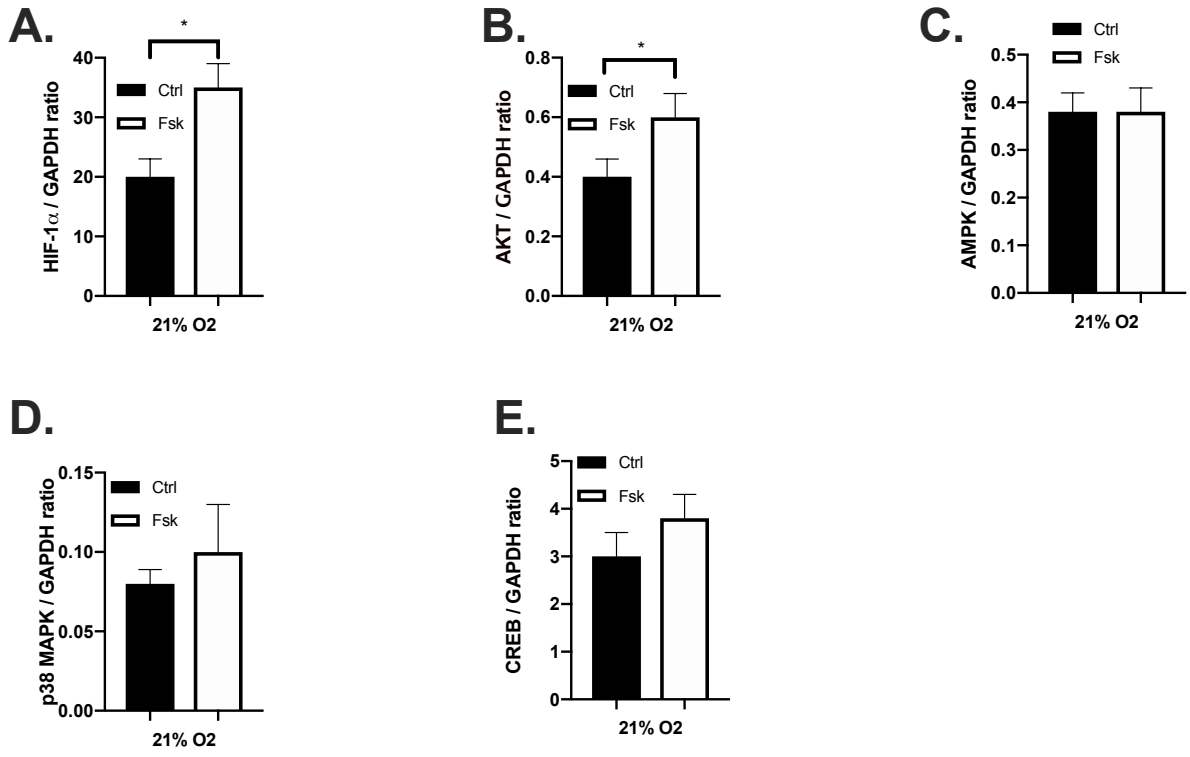


Figure 121

Forskolin increases HIF-1a and AKT expression in high oxygen tension at 24h. Forksolin has no effect on AMPK, p38 MAPK, CREB expression.

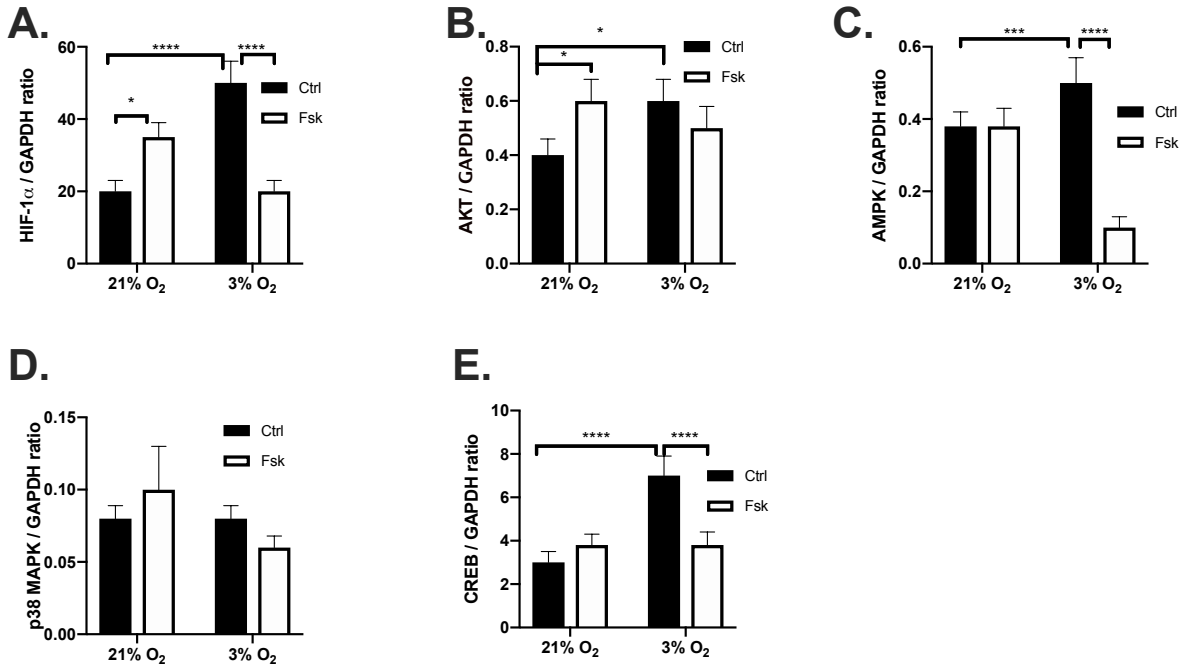


Figure 122

Hypoxia increases HIF-1a, AKT, AMPK, CREB expression at 24h. Hypoxia reverses the effect of forskolin on HIF-1a expression and abolishes the effect of forskolin on AKT expression. AMPK expression is reduced by forskolin under low

oxygen tension. CREB expression appears to be reduced by forskolin under low oxygen tension, but the basal levels of CREB is increased under low oxygen tension.

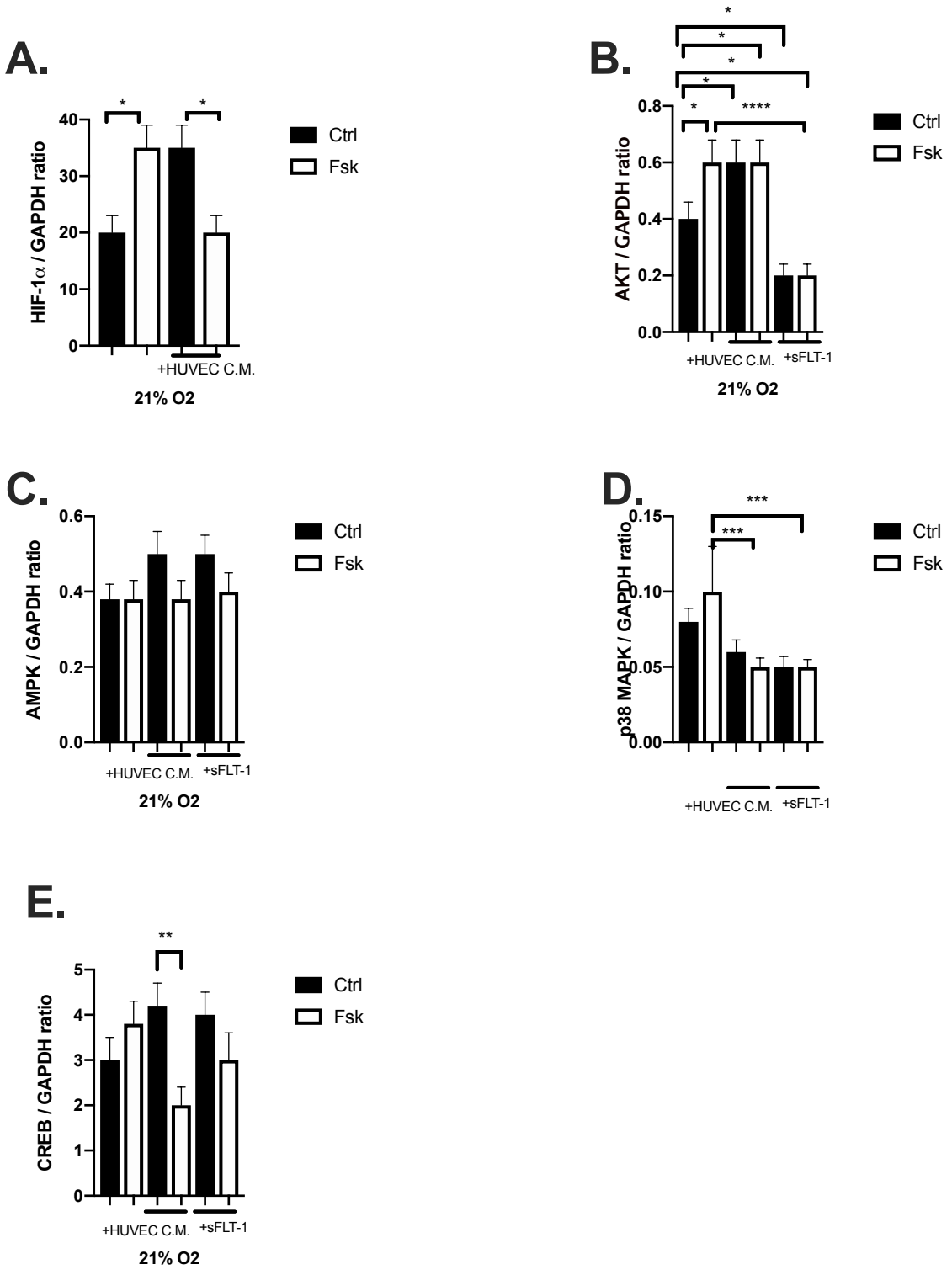


Figure 123

Conditioned media from HUVEC increases HIF-1a in undifferentiated BEWO cells. This effect is lost in differentiated BEWO cells. Forskolin increases AKT expression. Conditioned media from HUVEC increases AKT in undifferentiated BEWO cells. No further change in AKT expression is seen when conditioned media from HUVEC is added to differentiated BEWO cells. sFLT-1 reduces AKT expression. The ability of forskolin to increase AKT is lost in presence of sFLT-1.No effect on AMPK expression is seen in undifferentiated and differentiated BEWO cells. Conditioned media from HUVEC and sFLT-1 has no

effect on AMPK in undifferentiated and differentiated BEWO cells. Conditioned media from HUVEC and sFLT-1 reduces p38 MAPK in differentiated BEWO cells.

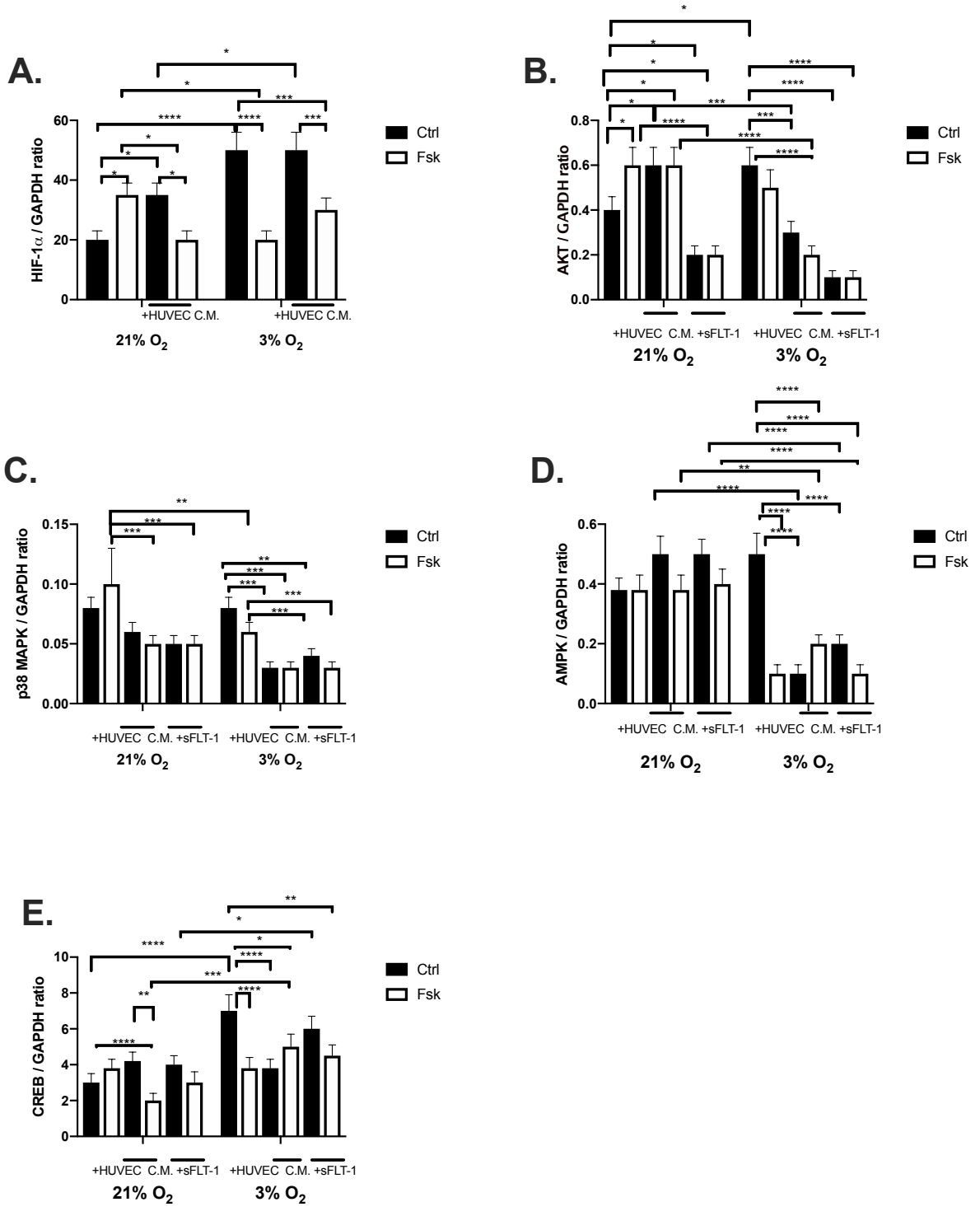


Figure 124

Under high oxygen tension, forskolin increases HIF-1a expression. Conditioned media from HUVEC increases HIF-1a protein expression in undifferentiated BEWO cells. Conditioned media from HUVEC decreases HIF-1a expression in differentiated BEWO cells. Hypoxia increases HIF-1a expression. Forskolin reduces HIF-1a expression. Conditioned media from HUVEC has no effect on HIF-1a expression in undifferentiated and differentiated BEWO cells in hypoxia.

Under high oxygen tension, forskolin increases AKT expression. Conditioned media from HUVEC increases AKT expression. Conditioned media from HUVEC has no effect on AKT in differentiated BEWO cells. sFLT-1 decreases AKT expression in undifferentiated and differentiated BEWO cells. Hypoxia increases AKT protein expression. Under low oxygen tension, conditioned media from HUVEC as well as sFLT-1 decrease AKT expression in undifferentiated and differentiated BEWO cells.
Forskolin has no effect on p38 MAPK expression in undifferentiated BEWO cells in high oxygen tension. Conditioned media from HUVEC / sFLT-1 has no effect on p38 MAPK expression in undifferentiated and differentiated BEWO cells in high oxygen tension. Hypoxia has no effect on p38 MAPK expression. Forskolin has no effect on p38 MAPK expression. In hypoxia, conditioned media from HUVEC decreases p38 MAPK expression in undifferentiated and differentiated BEWO cells. In hypoxia, sFLT-1 decreases p38 MAPK expression in undifferentiated and differentiated BEWO cells. In high oxygen tension,

Forskolin has no effect on AMPK protein expression. Conditioned media from HUVEC has no effect on AMPK expression in undifferentiated and differentiated BEWO cells. sFLT-1 has no effect on AMPK expression in undifferentiated and differentiated BEWO cells. Hypoxia has no effect on AMPK expression. Forskolin reduces AMPK protein expression. Conditioned media from HUVEC and sFLT-1 reduces AMPK expression in undifferentiated BEWO cells but remains unaffected in differentiated BEWO cells.

Forskolin has no effect on CREB protein expression. Conditioned media from HUVEC and sFLT-1 has no effect on CREB protein expression. Conditioned media from HUVEC reduces CREB protein expression in differentiated BEWO cells.
sFLT-1 has no effect on CREB expression in differentiated BEWO cells. Hypoxia increases CREB protein expression.
In low oxygen tension, forskolin reduces CREB protein expression. Conditioned media from HUVEC reduces CREB expression in undifferentiated BEWO cells but has no effect in differentiated BEWO cells. sFLT-1 has no effect on CREB expression in undifferentiated and differentiated BEWO cells.

Table 51

	HIF-1a		AKT		P38 MAPK		AMPK		CREB	
	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2
Ctrl	NS	↑	NS	↑	NS	NS	NS	NS	NS	↑
Fsk	↑	↓	↑	NS	NS	NS	NS	↓	NS	↓
HUVEC C.M.	↑	NS	↑	↓	NS	↓	NS	↓	NS	↓
HUVEC C.M. + Fsk	↓	↑	NS	↓	↓	↓	NS	NS	↓	NS
SFLT-1	NS	NS	↓	↓	NS	↓	NS	↓	NS	NS
SFLT-1 + Fsk	NS	NS	NS	NS	↓	↓	NS	NS	NS	NS

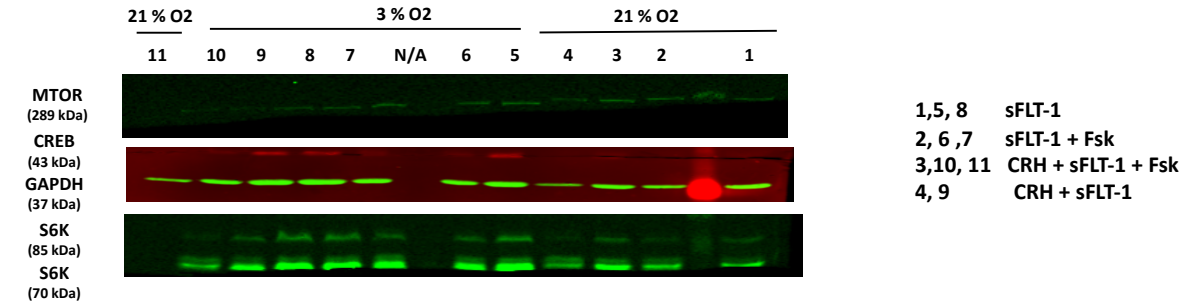


Figure 125

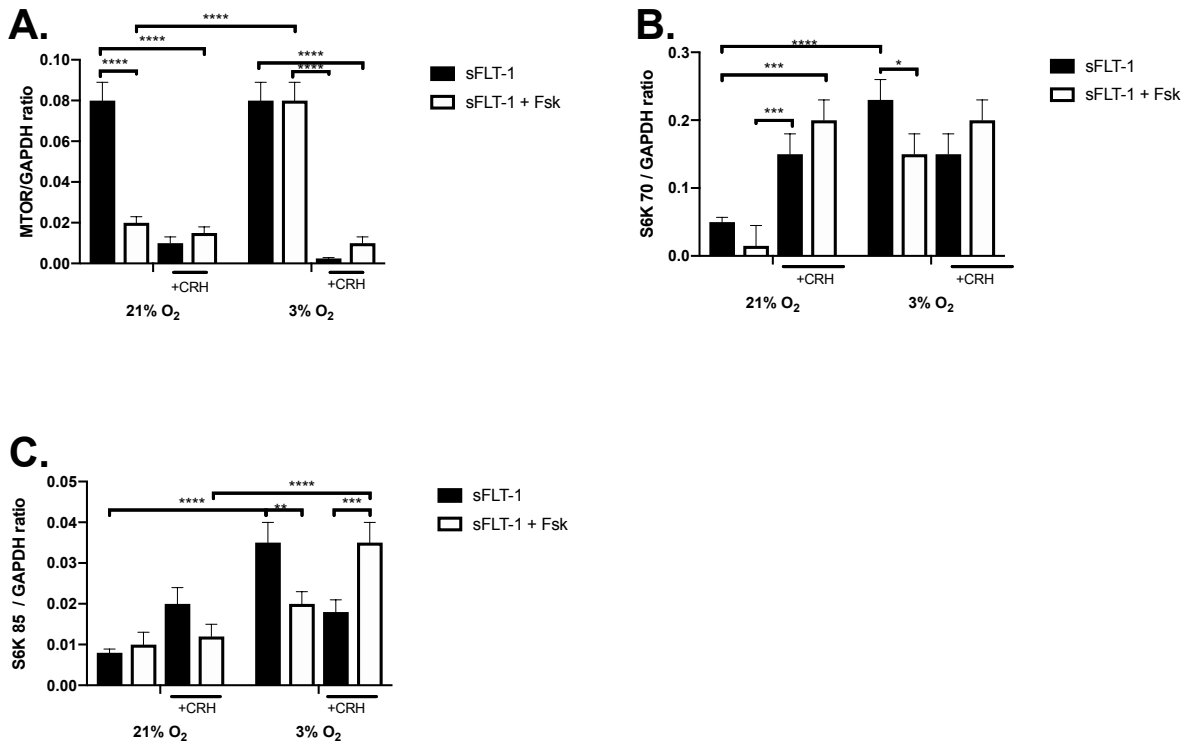


Figure 126

Table 52

24H	MTOR		S6K70		S6K85	
	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2
SFLT-1	NS	NS	NS	↑	NS	↑
SFLT-1 + Fsk	↓	NS	NS	↓	NS	↓
CRH + SFLT-1	↓	↓	↑	↓	NS	↓

CRH + SFLT-1 + Fsk	NS	↓	↑	NS	NS	↑

Bewo 24h

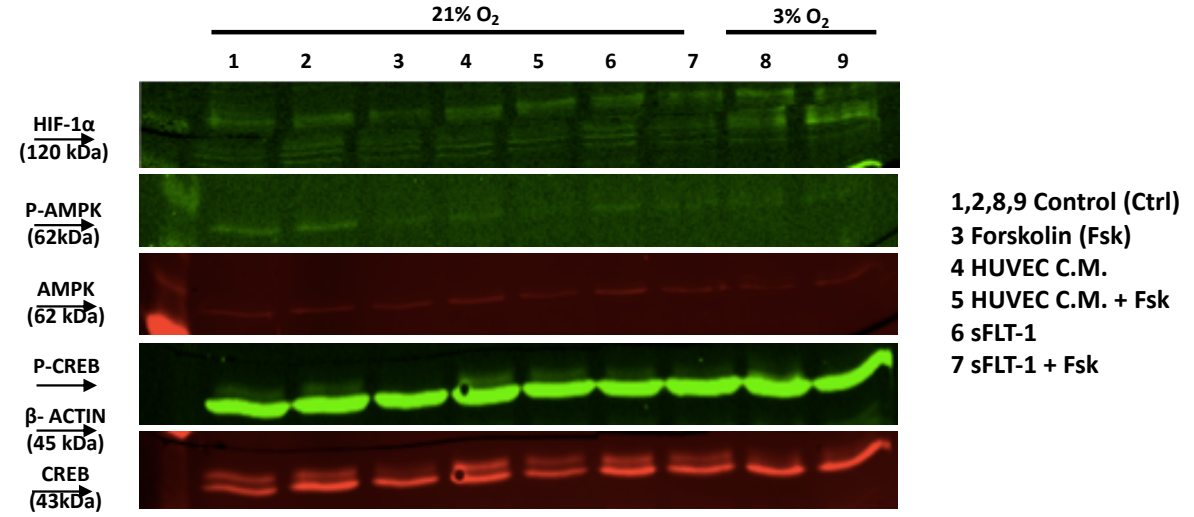


Figure 127

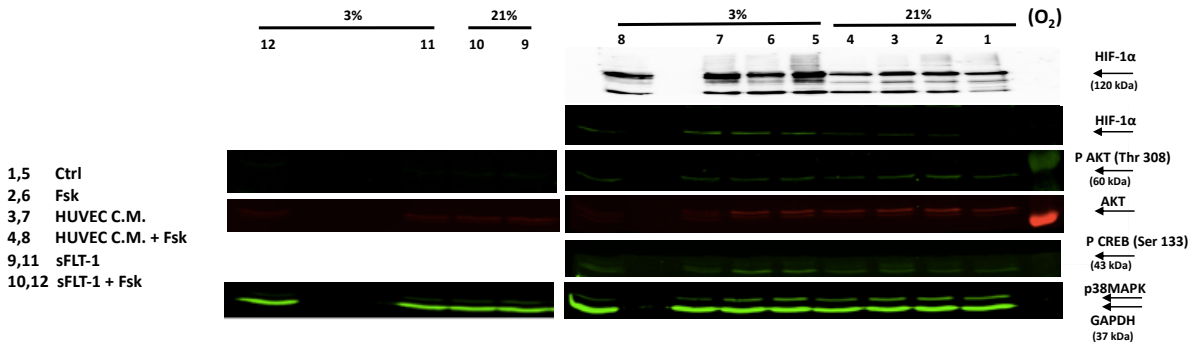


Figure 128

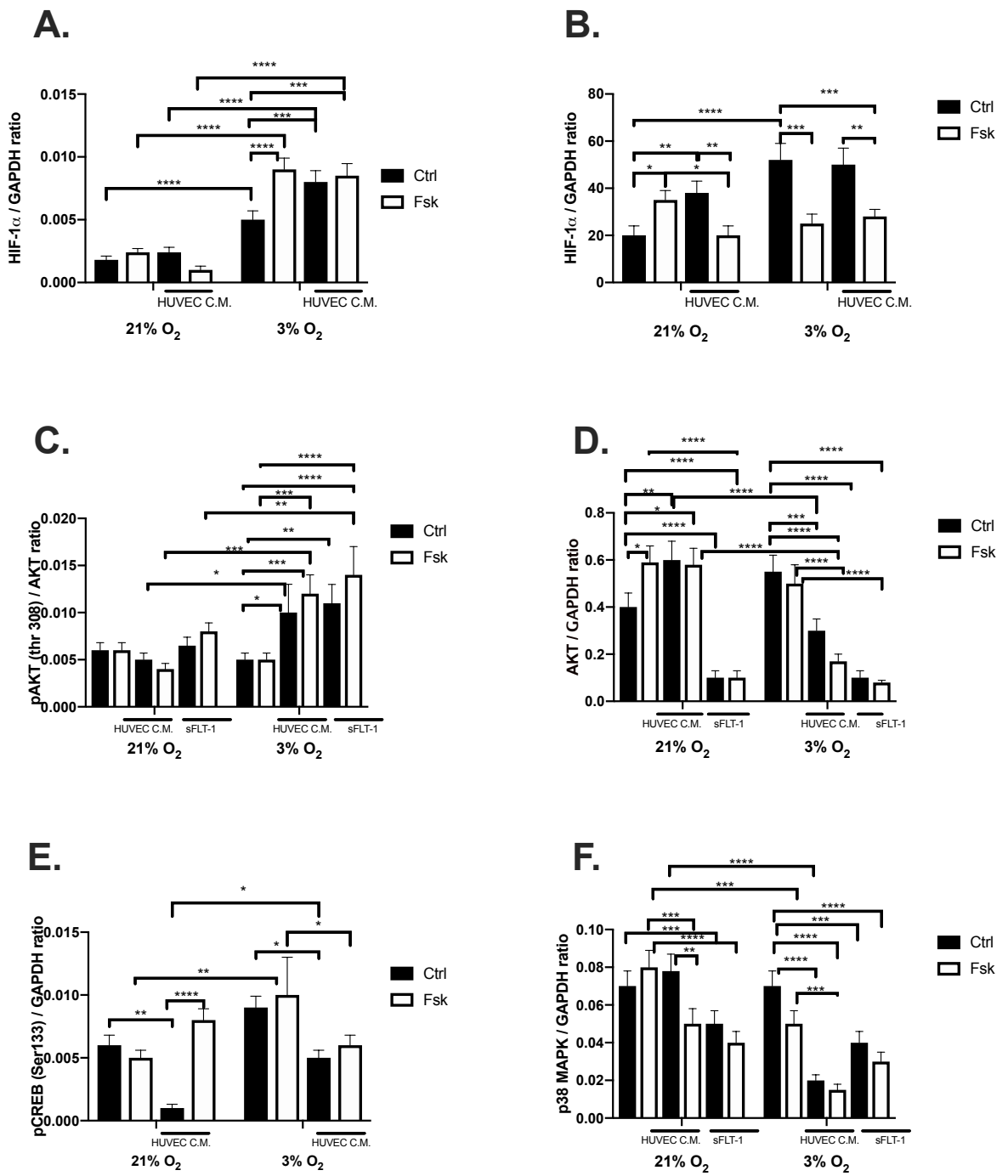


Figure 129

LICOR analysis of HIF-1a expression shows that hypoxia increases HIF-1a , which is further upregulated in differentiated BEWO cells IN 24H. Conditioned media from HUVEC increases HIF-1a . ECL analysis shows Forskolin increases HIF-1a expression. Conditioned media from HUVEC increases HIF-1a expression. The expression of HIF-1a is brought back to basal levels when conditioned media from HUVEC is added to differentiated BEWO cells.

ECL analysis shows hypoxia increases HIF-1a expression.Hypoxia reverses the effect of forskolin on HIF-1a expression. Hypoxia abolishes the effect of conditioned media from HUVEC on HIF-1a in undifferentiated and differentiated BEWO cells. Under high oxygen tension, forskolin has no effect on pAKT expression. Conditioned media from HUVEC and sFLT-1 has no effect on pAKT in undifferentiated and differentiated BEWO cells.

Hypoxia has no effect on pAKT expression.Forskolin has no effect on pAKT expression in high and low oxygen tension.Conditioned media from HUVEC and sFLT-1 increases pAKT (thr 308) expression in undifferentiated and differentiated BEWO cells in low oxygen tension.

Forskolin increases AKT expression in high oxygen tension.Conditioned media from HUVEC increase AKT expression in undifferentiated BEWO cells but not in differentiated BEWO in high oxygen tension.sFLT-1 decreases AKT expression in undifferentiated and differentiated BEWO cells in high oxygen tension.

Hypoxia increase AKT protein expression.Hypoxia abolishes the effect of forskolin on AKT expression. Conditioned media from HUVEC decrease AKT protein expression in undifferentiated and differentiated BEWO cells. sFLT-1 decreases AKT expression in undifferentiated and differentiated BEWO cells.

Forskolin has no effect on pCREB expression in high and low oxygen tension.Conditioned media from HUVEC reduce pCREB expression. Conditioned media from HUVEC increases pCREB expression in differentiated BEWO cells.
Hypoxia increases pCREB expression.Conditioned media from HUVEC reduce pCREB expression in hypoxia in undifferentiated and in differentiated BEWO cells.

Conditioned media from HUVEC has no effect on p38 MAPK expression in undifferentiated BEWO cells but reduce p38 MAPK expression in differentiated BEWO cells in high oxygen tension. sFLT-1 reduce p38 MAPK expression in undifferentiated and differentiated BEWO cells in high oxygen tension.Conditioned media from HUVEC and sFLT-1 reduce p38 MAPK protein expression in undifferentiated and differentiated BEWO cells in low oxygen tension.

Table 53

	HIF-1a		HIF-1a (ECL)		PAKT /AKT		AKT/GAPDH		PCREB		P38 MAPK	
	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2	21% O2	3% O2
Ctrl	NS	↑	NS	↑	NS	NS	NS	NS	NS	NS	NS	NS
Fsk	NS	↑	↑	↓	NS	NS	↑	NS	NS	NS	NS	NS
HUVEC C.M.	NS	↑	↑	NS	NS	↑	↑	↓	↓	↓	NS	↓
HUVEC C.M. + Fsk	NS	NS	↓	NS	NS	↑	NS	↓	NS	↓	↓	↓
SFLT-1	NS	NS	NS	NS	NS	↑	↓	↓	NS	NS	↓	↓
SFLT-1 + Fsk	NS	NS	NS	NS	NS	↑	↓	↓	NS	NS	↓	↓

6.1.35 Endogenous secretion of PLGF, VEGF and sFLT – 1 in BEWO cells over 48h under high oxygen tension

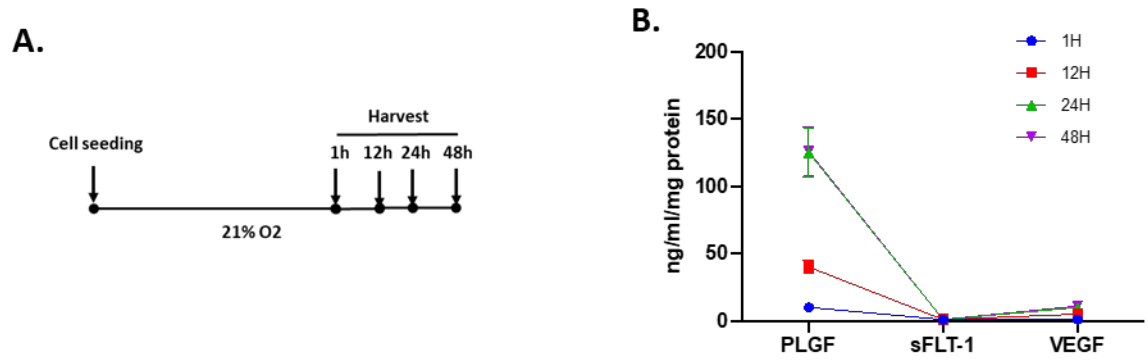


Figure 130

*Under high oxygen tension, PLGF and VEGF are endogenously secreted in a time dependent manner upto 48h in BEWO cells, the former being released in excess. (A) Experiment protocol, where after 80% confluency, fresh media was added and supernatants were collected at different time points and MSD ELISA assay was done on PLGF, SFLT-1 and VEGF proteins. (B) Both PLGF and VEGF proteins were significantly released in a time dependent manner upto 48 h from BEWO cells at 21% O2, the former being released in higher amounts. SFLT-1 protein was released in minimal amounts endogenously. p=0.1234 ns ; * 0.033; ** .002; *** .0002; ****< .0001; n=3.Data expressed as ng/ml normalised against mg protein. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

The endogenous release of PLGF, VEGF and sFLT-1 were analysed at various time points upto 48h under high oxygen tension using MSD assay. BEWO cells were incubated with F12K culture media and the supernatants were assessed. BEWO secretes PLGF protein in excess amounts (upto 45 ng/ml/mg protein) compared to other molecules such as sFLT-1 (1ng/ml/mg protein) and VEGF (upto 15 ng /ml/mg protein). PLGF is endogenously secreted by BEWO cells in a time dependent manner. Similar pattern of secretion is seen with VEGF. sFLT-1 is secreted in a steady manner throughout 48 hours.

6.1.36 Influence of oxygen tension on the pattern of secretion of PLGF in BEWO cells over 24h

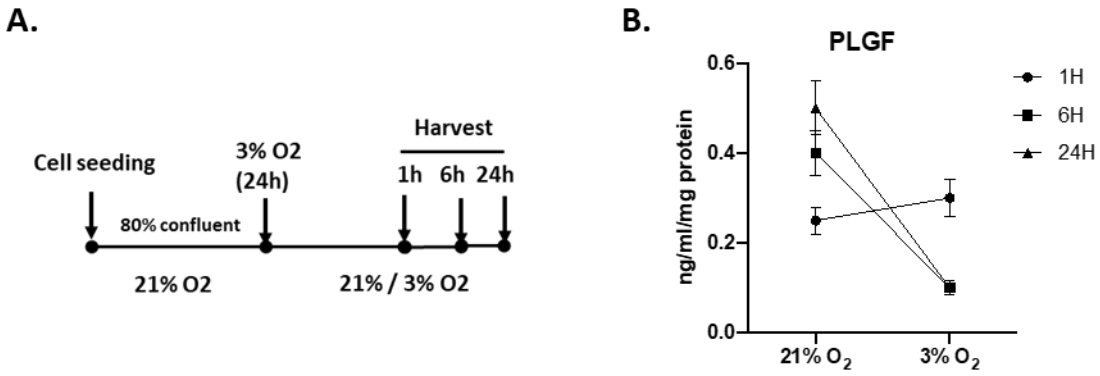


Figure 131

*Low oxygen tension reverses the time dependent increase of PLGF release in BEWO cells. (A) Experiment protocol, where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Supernatants were collected at different time points and MSD ELISA assay was done on PLGF protein. (B) The pattern of secretion of PLGF in BEWO cells was reversed when cells were exposed to 3%O2. p=0.1234 ns ; * 0.033; ** .002 ;*** .0002 ;****< .0001; n=3.Data expressed as ng/ml normalised against mg protein. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

The influence of oxygen tension on PLGF release in BEWO cells was investigated at various time points, upto 24h, using MSD assay. PLGF release was seen in a time dependent manner upto 24h under high oxygen tension. While similar amount of PLGF is released at 1h under both high and low oxygen tension, the secretion of PLGF was reduced by 6h upto 66% under low oxygen tension, reaching a saturation by 24h.

6.1.37 The regulation of PLGF release by CRH in BEWO cells over 24h under high and low oxygen tension

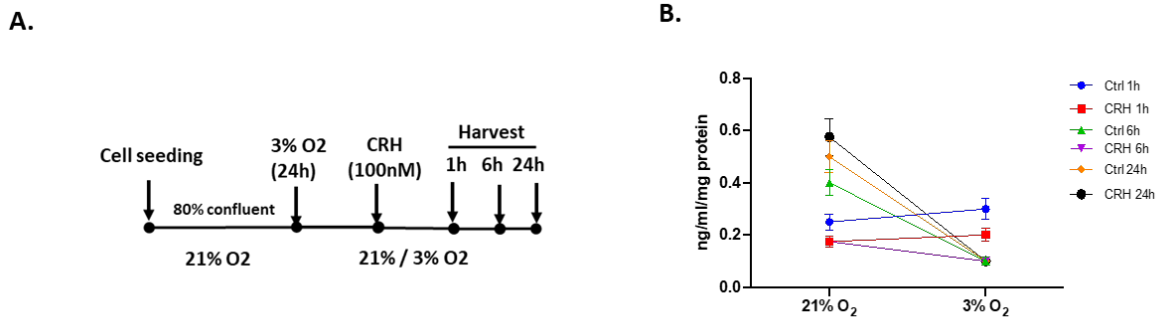


Figure 132

*Under high oxygen tension, CRH reduces PLGF release at 6h but has no effect at other time points. Under low oxygen tension, CRH does not regulate PLGF release in BEWO cells. (A) Experiment protocol, where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were further treated with 100nM CRH and supernatants were collected at different time points and MSD ELISA assay was done on PLGF protein. (B) CRH significantly reduced PLGF protein at 6h at 21% O2 but had no effect on PLGF protein at other time points at both 21 % O2 and 3% O2. p=0.1234 ns ; * 0.033; ** .002 ;*** .0002 ;****< .0001; n=3.Data expressed as ng/ml normalised against mg protein. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

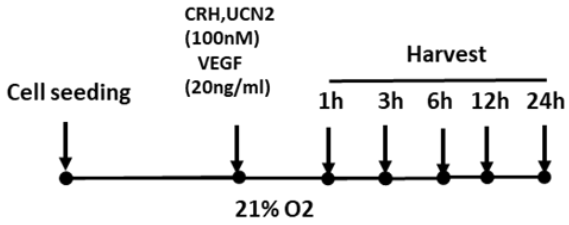
Table 54

PLGF release in BEWO	21% O2		3% O2	
	Ctrl	CRH	Ctrl	CRH
1H	NS	NS	NS	↓
6H	↑	↓	↓	NS
24H	NS	NS	NS	NS

Under high and low oxygen tension, the regulation of PLGF release by CRH was studied at different time points, upto 24h, using MSD assay. Under high oxygen tension, CRH reduced PLGF release at 6h, but had no effect at other time points. Under low oxygen tension, the ability to reduce PLGF release at 6h by CRH is lost.

6.1.38 The regulation of secretion of PLGF and VEGF in BEWO cells by CRH, UCN2, VEGF over 24h under high oxygen tension

1.



2.

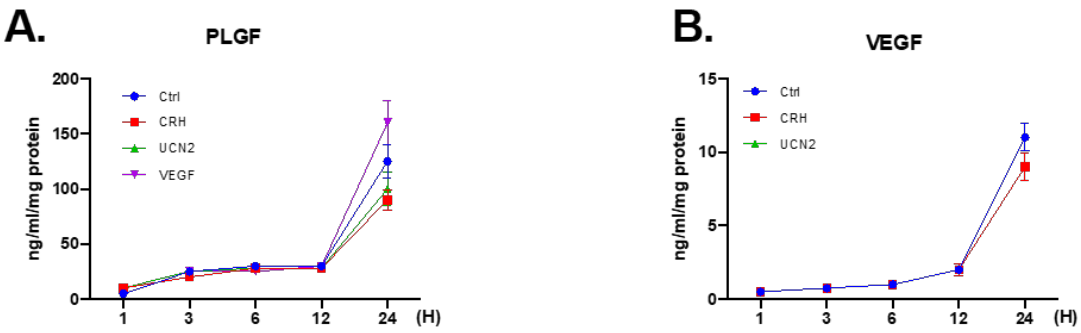


Figure 133

*Under high oxygen tension, while VEGF increased PLGF release at 24h, CRH and UCN2 decreased PLGF release at 24h. (A) Experiment protocol, where after 80% confluency, fresh media was added and cells were further treated with 100nM CRH, 100nM UCN2 or 20ng/ml VEGF and supernatants were collected at different time points and MSD ELISA assay was done on PLGF and VEGF proteins. (B) PLGF protein secretion was significantly reduced by both CRH and UCN2 and increased by VEGF at 24h at 21% O2 in BEWO cells. p=0.1234 ns ; * 0.033; ** .002 ;*** .0002 ;****< .0001; n=3.Data expressed as ng/ml normalised against mg protein. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

Table 55

PLGF release in BEWO	21% O2			
	Ctrl	CRH	UCN2	VEGF
1H	NS	NS	NS	NS
3H	NS	NS	NS	NS
6H	NS	NS	NS	NS
12H	NS	NS	NS	NS
24H	NS	↓	↓	↑

Table 56

VEGF release in BEWO	21% O2			
	Ctrl	CRH	UCN2	
1H	NS	NS	NS	
3H	NS	NS	NS	
6H	NS	NS	NS	
12H	↑	NS	NS	
24H	↑	↓	↓	

To verify the influence of CRH, UCN2 and VEGF on the pattern of secretion of PLGF and VEGF in BEWO cells over the period of 24h, the BEWO cells were treated with 100nM of CRH, 100nM of UCN2 and 20ng/ml VEGF over the period of 24h and supernatants were collected at different time points and PLGF and VEGF were measured using MSD ELISA assay. CRH and UCN2 decreased PLGF release at 24h. VEGF increased PLGF release at 24h.

Expression and release of PLGF in syncytialised BEWO cells under high oxygen tension

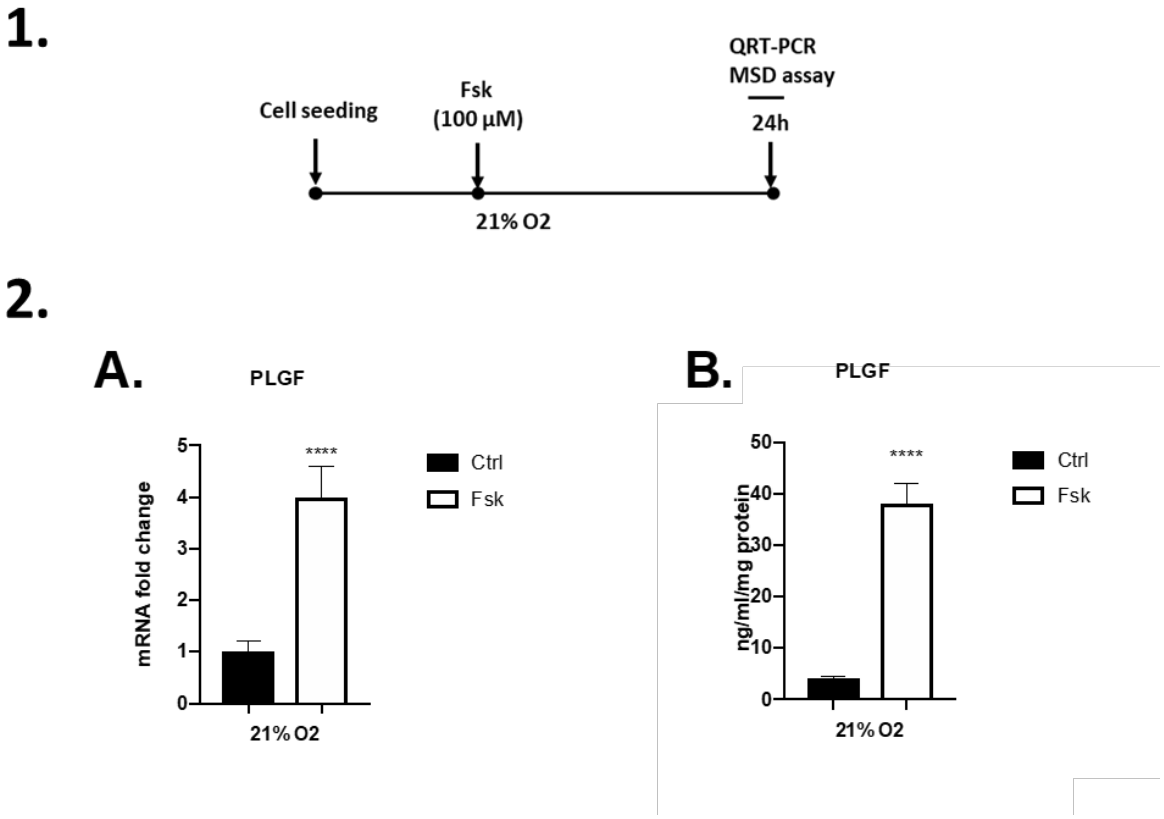


Figure 134

*CAMP/PKA signalling pathway increases PLGF mRNA expression and release from BEWO cells. 1. Experiment protocol. 2.(A) QRT-PCR analysis showed that the expression of PLGF MRNA was significantly upregulated and (B) MSD ELISA assay showed the PLGF protein secretion is significantly higher in BEWO cells treated with 100um forskolin for 24h at 21% O2. **** p<0.0001; n=3; Fsk: forskolin. Data expressed as (A) fold change of gene expression, relative to control 24h, after normalising against B-actin and (B) ng/ml normalised against mg protein. Data were analysed using a two-tailed unpaired t-test.*

The expression of PlGF messenger RNA (mRNA) was analysed using real-time PCR in BEWO cells. Glyceraldehyde 3-phosphate dehydrogenase and B actin were used as internal controls, with the latter proving to be more consistent, as reported previously https://www.ncbi.nlm.nih.gov/pubmed/14706621.[22] To verify the activation of the cAMP-PKA pathway, BEWO cells, a well characterised model of trophoblasts, were treated with 100 µM forskolin (a strong activator of adenylate cyclase) for 24h. Under high oxygen tension (21%O2), forskolin induced upregulation of PLGF mRNA expression by 4 fold compared to control.

To verify that changes in gene expression were reflected by protein synthesis, supernatants from cells treated with the same compound were subjected to a specific PlGF ELISA (figure). In BEWO cells, forskolin increased PlGF protein by >35-fold, after 24 hours.

6.1.39 The regulation of expression and release of PLGF in syncytialised BEWO cells by low oxygen tension

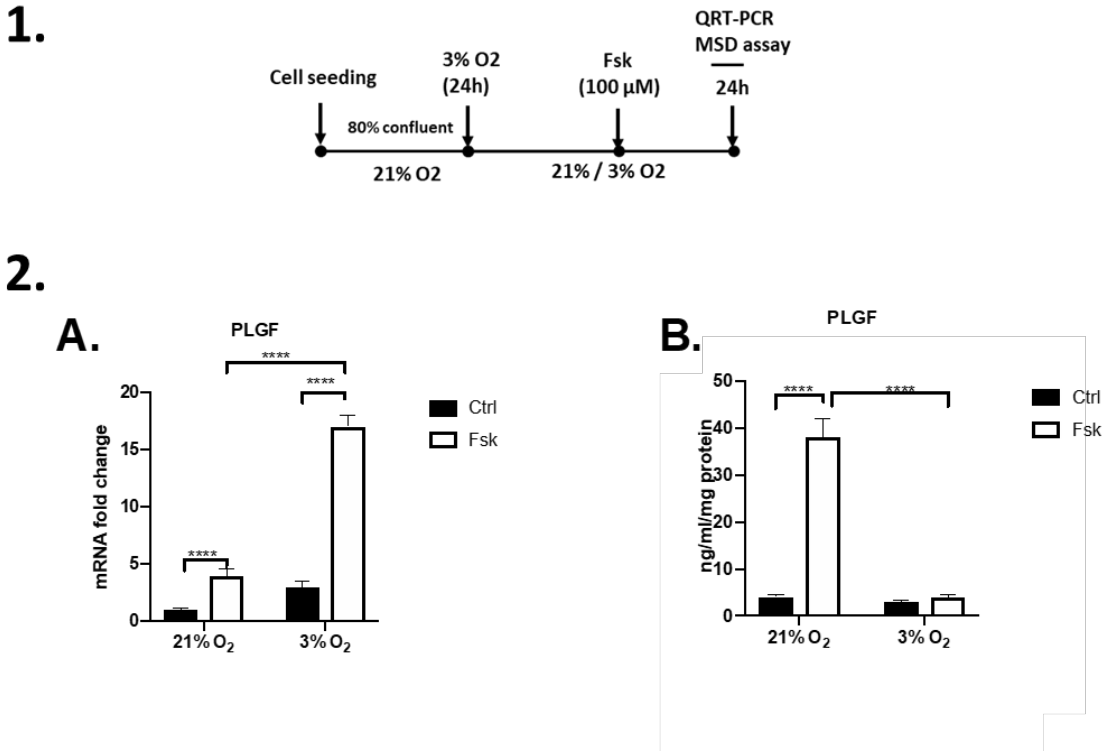


Figure 135

*Low oxygen tension increases PLGF mRNA expression but reduces PLGF release in syncytialised BEWO cells. 1. Experiment protocol; 2.(A) QRT-PCR analysis showed that the expression of PLGF MRNA was significantly upregulated and (B) MSD ELISA assay showed the PLGF protein secretion is significantly reduced in BEWO cells treated with 100Um forskolin for 24h at 3% O2. **** p<0.0001; n=3; Fsk: forskolin. Data expressed as (A) fold change of gene expression, relative to control 24h, after normalising against B-actin and (B) ng/ml normalised against mg protein. Data were analysed using a two-tailed unpaired t-test.*

To verify the regulation of expression and release of PLGF by low oxygen tension (3%O2), BEWO cells were incubated under high (21% O2) and low (3% O2) oxygen tensions for 24h and mRNA and protein release of PLGF were analysed using QRT-PCR and ELISA assay. The cells were further incubated with forskolin. Under 3% O2, PLGF mRNA was upregulated by <3 fold. This is however not reflected by protein release. Low oxygen tension increased forskolin induced PLGF mRNA by 12 fold but reduced PLGF by 70% at the protein secretion level.

The regulation of expression and release of PLGF by CRH in BEWO cells under high and low oxygen tension

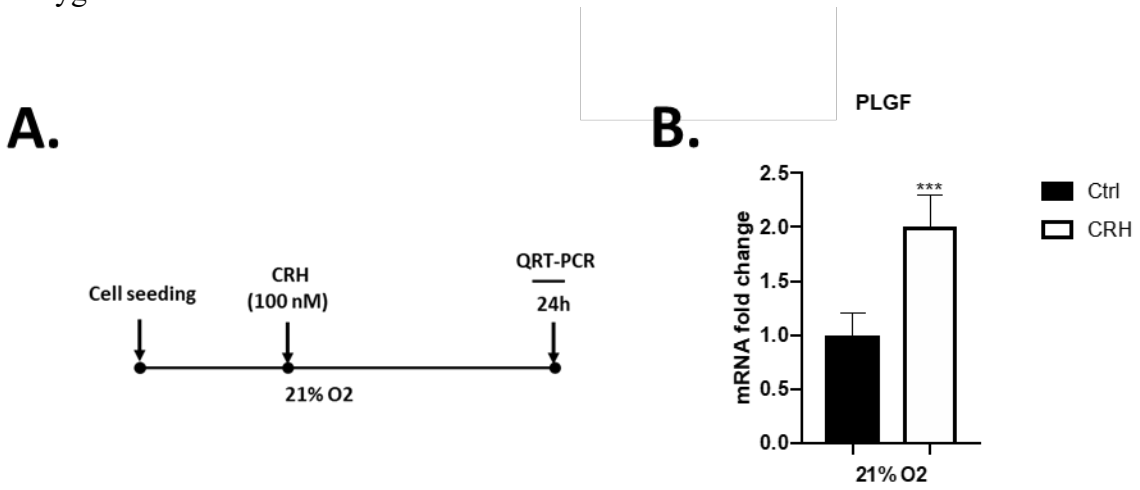


Figure 136

*CRH increases PLGF mRNA expression under high oxygen tension. A) Experiment protocol; B) QRT-PCR analysis showed that the expression of PLGF MRNA was significantly upregulated in BEWO cells treated with 100nm CRH for 24h at 21% O2. *** p=0.0002; n=3; CRH: corticotrophin releasing hormone. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.*

The regulation of expression of PLGF by CRH was investigated in BEWO cells by incubating cells with 100nM CRH under high oxygen tension and PLGF mRNA was analysed using QRT-PCR. CRH increases PLGF mRNA expression by 2 fold under high oxygen tension.

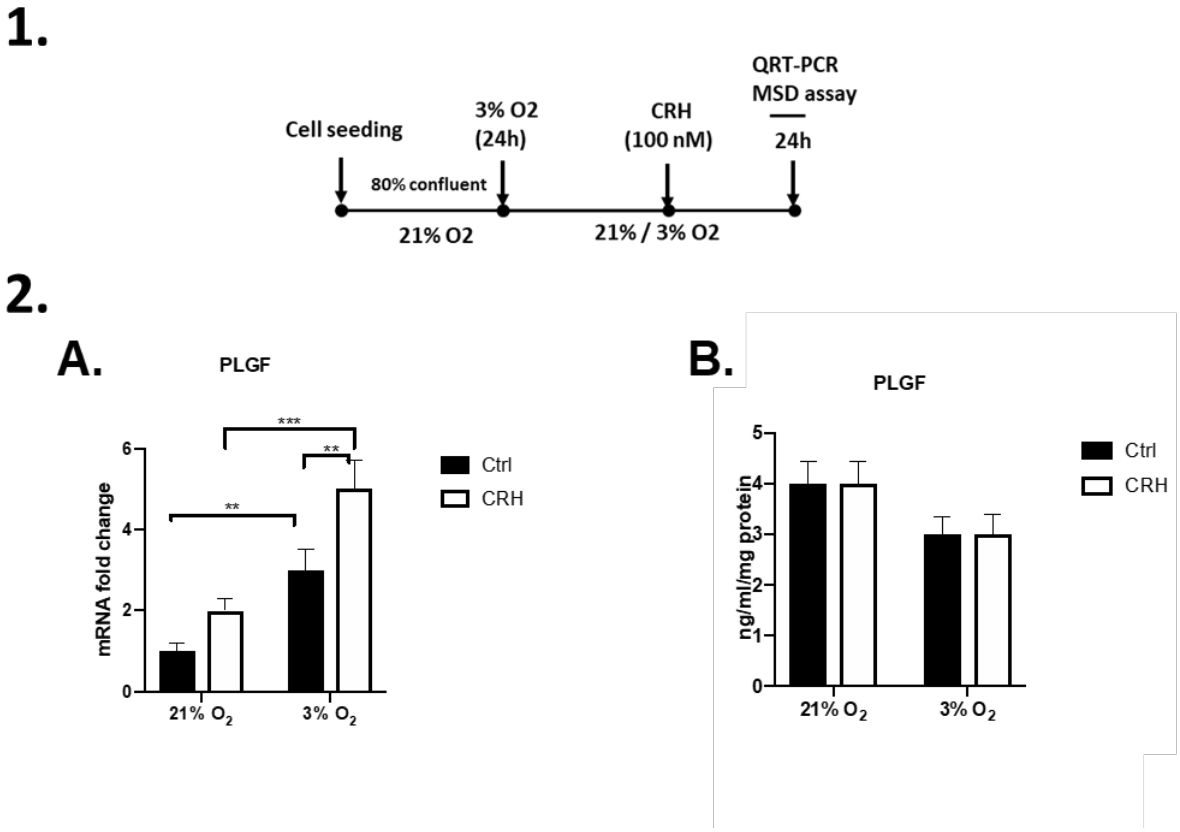


Figure 137

*Low oxygen tension increases PLGF mRNA expression but not release. CRH further potentiates the actions of hypoxia on PLGF mRNA expression but not reflected at protein release level. 1. Experiment protocol; 2. (A) QRT-PCR analysis showed that the expression of PLGF MRNA was significantly upregulated and (B) MSD ELISA assay showed the PLGF protein secretion was unaffected in BEWO cells exposed to 3% O2 for 24h. BEWO cells further treated with 100NM CRH showed that CRH further potentiates the actions of hypoxia on the (A) expression of PLGF MRNA but no effect was seen at the (B) protein secretion level. p=** .002 ;*** .0002 ;****< .0001; **** p<0.0001; n=3; CRH : corticotrophin releasing hormone. Data expressed as (A) fold change of gene expression, relative to control 24h, after normalising against B-actin and (B) ng/ml normalised against mg protein. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

The regulation of expression and release of PLGF by CRH was investigated in BEWO cells under low oxygen tension by incubating cells with 100nM CRH and PLGF mRNA and release was analysed using QRT-PCR and ELISA. Hypoxia increases PLGF mRNA by 3 fold and CRH potentiates PLGF mRNA expression by 2 fold. This is not reflected at protein release level.

6.1.40 The regulation of Expression and release of PLGF by CRH in syncytialised BEWO cells under high oxygen tension

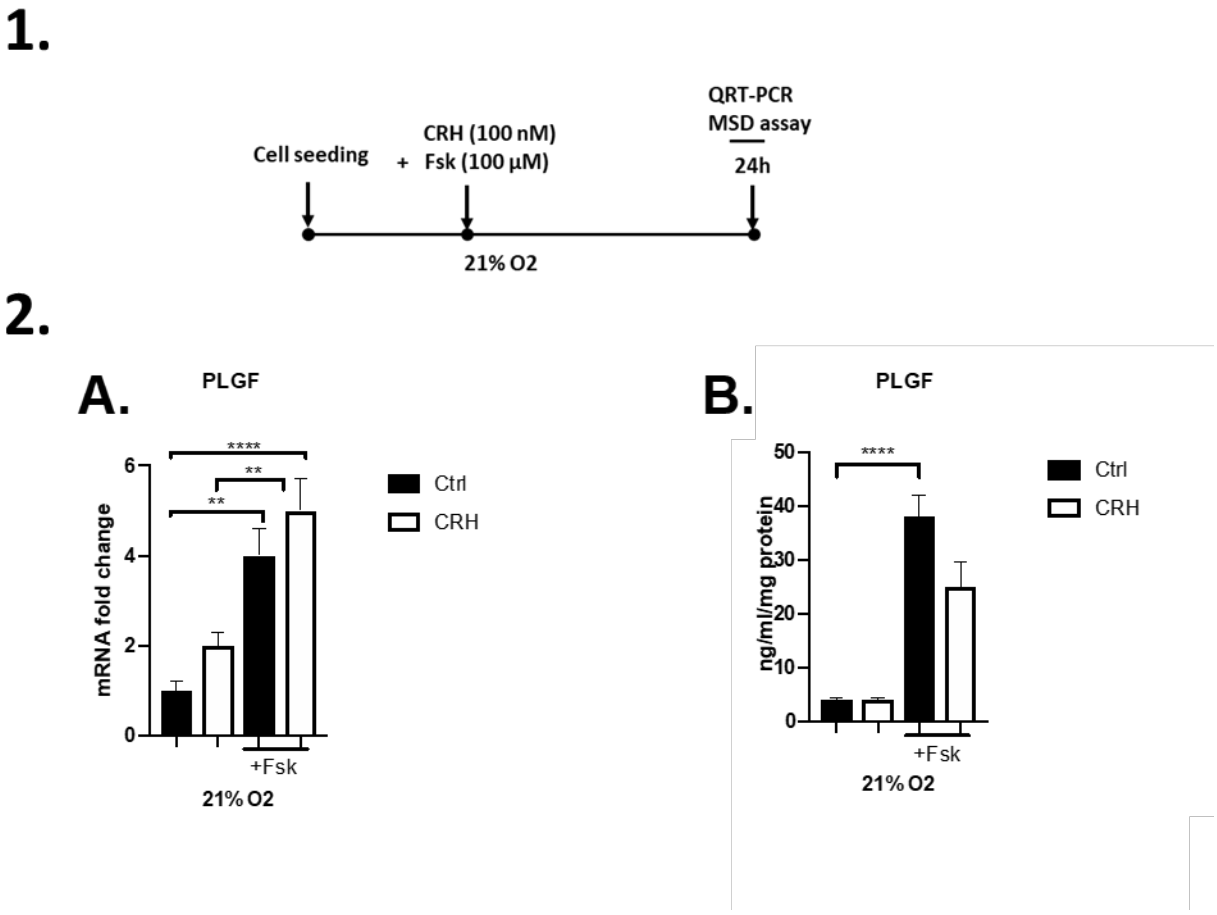


Figure 138

*CRH does not regulate forskolin induced PLGF mRNA expression and protein secretion under high oxygen tension in BEWO cells. 1. Experiment protocol; 2. (A) QRT-PCR analysis showed that the expression of PLGF MRNA and (B) MSD ELISA assay showed the PLGF protein secretion were unaffected in BEWO cells treated with 100nm CRH and 100um forskolin, exposed at 21% O2 for 24h. p=** .002 ;*** .0002 ;****< .0001; **** p<0.0001; n=3; Fsk: forskolin; CRH: corticotrophin releasing hormone. Data expressed as (A) fold change of gene expression, relative to control 24h, after normalising against B-actin and (B) ng/ml normalised against mg protein. Data were analysed using a one-way ANOVA with Tukey's multiple comparison test.*

The regulation of PLGF MRNA and release by CRH in syncytialised BEWO cells under high oxygen tension was investigated. PlGF mRNA upregulation and protein release by forskolin remained unaffected in the presence of 100nM CRH.

6.1.41 The regulation of Expression and release of PLGF by CRH in syncytialised BEWO cells under high and low oxygen tension

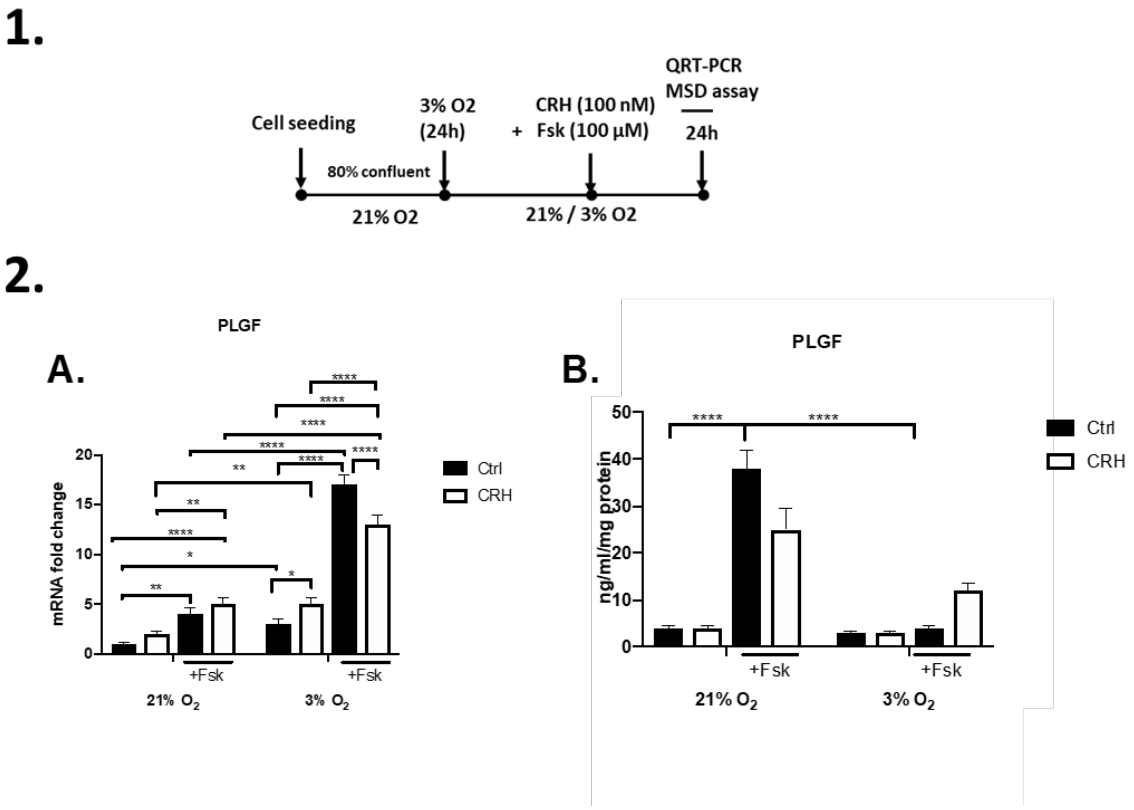


Figure 139

Under low oxygen tension, CRH upregulates PLGF mRNA but not protein release in BEWO cells and reduces PLGF mRNA in syncytialised BEWO cells. 1. *Experiment protocol; 2. (A) QRT-PCR analysis showed that the expression of PLGF MRNA was significantly upregulated and (B) MSD ELISA assay showed the PLGF protein secretion was unaffected in BEWO cells treated with 100NM CRH exposed at 3% O2 for 24h. Furthermore, some cells incubated with 100NM CRH along with 100um forskolin showed PLGF MRNA was downregulated and protein release remained unaffected.* p=* *0.033, ** .002 ;*** .0002 ; **** p<0.0001; n=3; CRH : corticotrophin releasing hormone; fsk: forskolin. Data expressed as (A) fold change of gene expression, relative to control 24h, after normalising against B-actin and (B) ng/ml normalised against mg protein. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

The regulation of PLGF mRNA and release by CRH in syncytialised BEWO cells under low oxygen tension was investigated. Under low oxygen tension, CRH upregulated PlGF mRNA by 2 fold. This was not reflected by protein secretion. PLGF mRNA upregulation by forskolin was reduced by 5 fold, in the presence of 100nM CRH. However, a trend towards increase in PLGF release was seen.

6.1.42 The regulation of release of PLGF by CRH via CRHR1 in BEWO cells under high oxygen tension

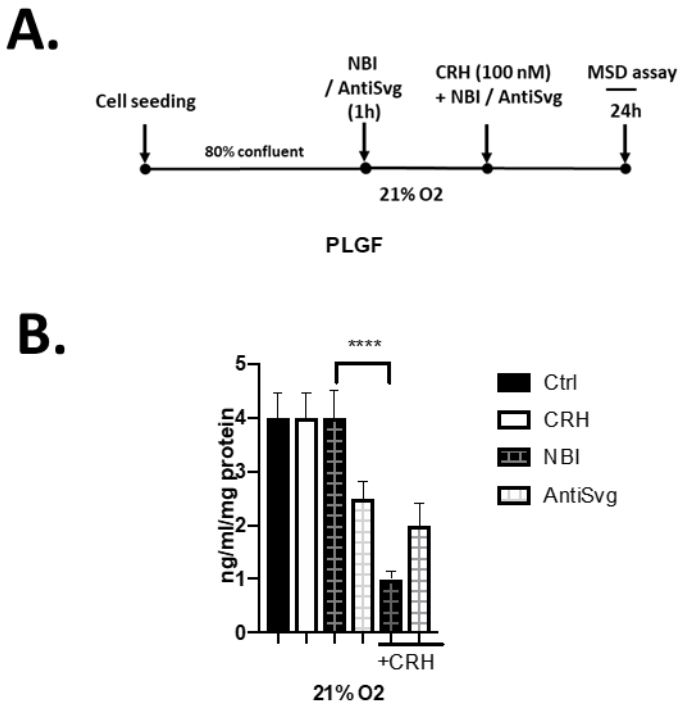


Figure 140

Under high oxygen tension, blocking CRHR1 reduces CRH mediated PLGF release. A, Experiment protocol, where after 80% confluency, fresh media was added. Cells were pretreated with CRHR1 /2 antagonists, NBI 27914 or anti-sauvagine-30 for 1h. The cells were further treated with 100nM CRH for 24h and supernatants were collected and MSD ELISA assay was done on PLGF protein. B. MSD ELISA assay showed PLGF protein was not affected in BEWO cells treated with 100nM CRH exposed at 21% O2 at 24h. Cells treated with NBI along with CRH showed reduced release of PLGF. No effect was seen in cells treated with anti-sauvagine 30 along with CRH. **** $p<0.0001$*; n=3; CRH : corticotrophin releasing hormone. Data expressed as ng/ml normalised against mg protein. Data were analysed using a one-way ANOVA with Tukey's multiple comparison test.*

The receptors involved in the regulation of release of PLGF by CRH were investigated in BEWO cells under high oxygen tension by incubating cells with 100nM CRH for 24h. Some cells were preincubated for 1 hour with, CRHR-1 and 2 antagonists, NBI-27914 and Anti-Sauvagine 30 respectively and PLGF release was analysed using ELISA. CRH did not affect PLGF release. PLGF release by CRH was inhibited by 80% in the presence of CRHR1 antagonist, NBI 27914. No effect is seen with CRHR2 antagonist, Anti-Sauvagine 30.

6.1.43 The regulation of release of PLGF by CRH via CRHR1 in BEWO cells under high and low oxygen tension

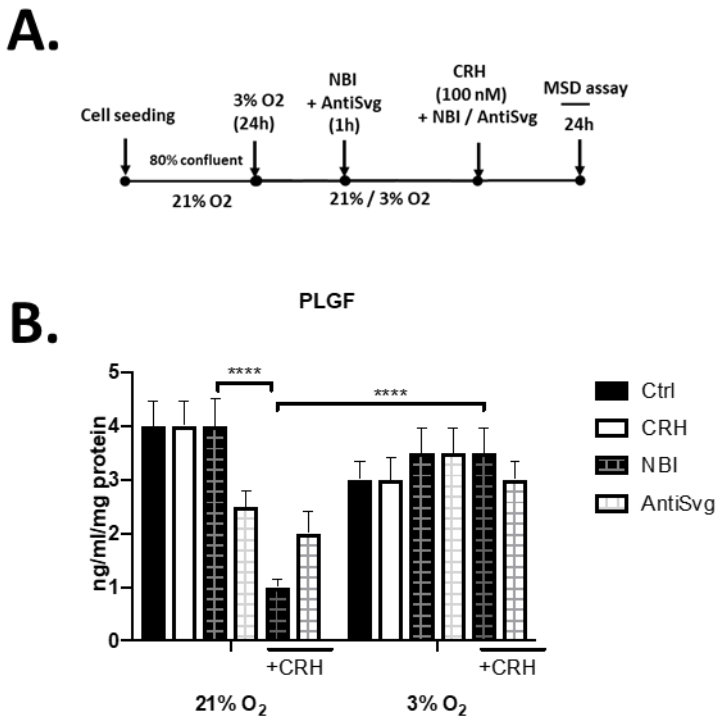


Figure 141

Under high but not low oxygen tension, blocking CRHR1 reduces CRH mediated PLGF release. A, Experiment protocol, , where after 80% confluency, fresh media was added and cells were exposed to 3% O2 for 24h. Cells were pretreated with CRHR1 /2 antagonists, NBI 27914 or anti-sauvagine-30 for 1h. The cells were further treated with 100nM CRH for 24h and supernatants were collected at 24h and MSD ELISA assay was done on PLGF protein. MSD ELISA assay showed under low oxygen tension, CRH has no effect on PLGF protein release. Treating cells with NBI 27914 or anti-sauvagine-30 along with CRH has no effect on PLGF release. **** p<0.0001*; n=3; CRH: corticotrophin releasing hormone. Data expressed as ng/ml normalised against mg protein. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

Table 57

PLGF release in BEWO	**21% O2**		**3% O2**	
	Ctrl	**CRH**	**Ctrl**	**CRH**
- NBI /AntiSvg-30	NS	NS	NS	NS
+ NBI	NS	↓	NS	NS
+ AntiSvg-30	NS	NS	NS	NS

The receptors involved in the regulation of release of PLGF by CRH was investigated in BEWO cells under high and low oxygen tension by incubating cells with 100nM CRH for 24h. Some cells were preincubated for 1 hour with, CRHR-1 and 2 antagonists, NBI-27914 and Anti-Sauvagine 30 respectively and PLGF release was analysed using ELISA. CRH did not affect PLGF release. Blocking CRHR1 or CRHR2 did not further affect CRH mediated PLGF release.

6.1.44 The regulation of release of PLGF by CRH via CRHR1 in syncytialised BEWO cells under high and low oxygen tension

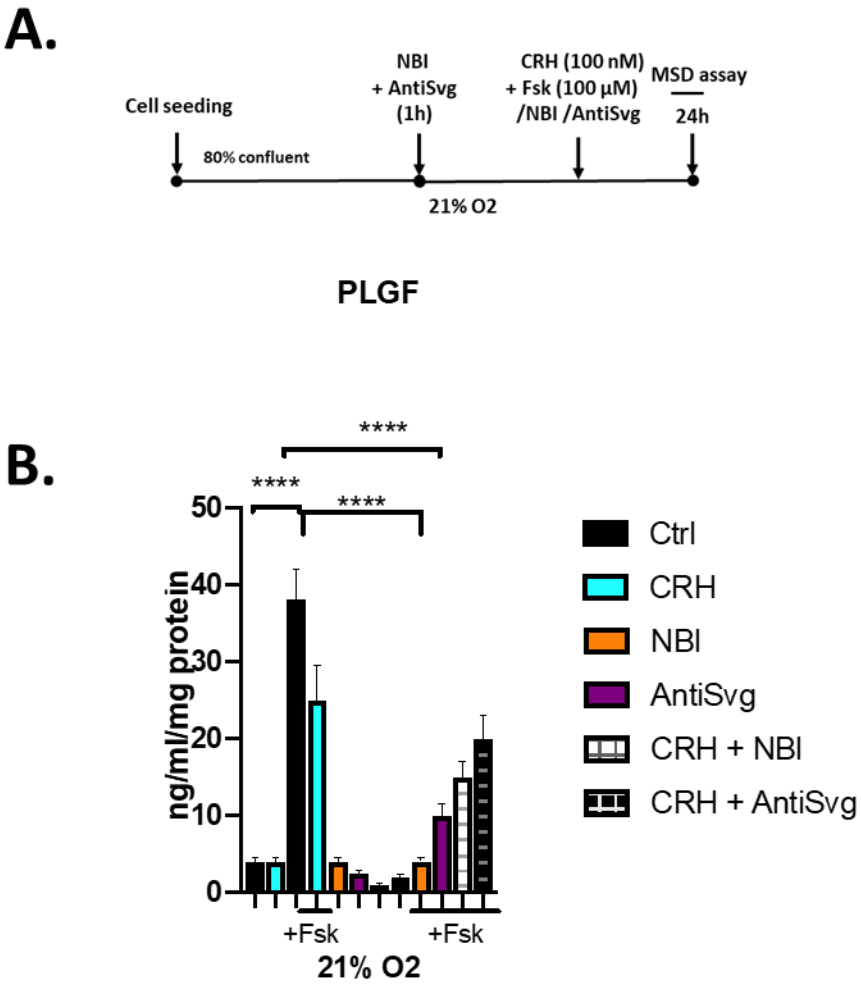


Figure 142

Under high oxygen tension,cAMP signalling pathway increases PLGF release via CRHR1. A, Experiment protocol, , where after 80% confluency, fresh media was added. Cells were pretreated with CRHR1 /2 antagonists, NBI 27914 or anti-sauvagine-30 for 1h. The cells were further treated with 100nM CRH along with 100um forskolin for 24h and supernatants were collected and MSD ELISA assay was done on PLGF protein. B. Cells incubated with 100um forskolin for 24h showed increase in PLGF release. Further combined treatment with NBI 27914 showed no increase in PLGF release. Cells treated with forskolin and antisauvagine – 30 showed trend towards an increase in PLGF release. A trend towards decrease in PLGF release was seen in cells treated with CRH and forskolin. Combined treatment with either NBI or anti-sauvagine 30 showed no effect on PLGF

release. **** p<0.0001*; n=3; CRH : corticotrophin releasing hormone. Data expressed as ng/ml normalised against mg protein. Data were analysed using a one-way ANOVA with Tukey's multiple comparison test.*

The receptors involved in the regulation of release of PLGF by CRH were investigated in syncytialised BEWO cells under high oxygen tension. BEWO cells under high oxygen tension were investigated by simultaneously incubating cells with 100nM CRH and 100µM forskolin for 24h. Some cells were preincubated for 1 hour with, CRHR-1 and 2 antagonists, NBI-27914 and Anti-Sauvagine 30 respectively and PLGF release was analysed using ELISA. CAMP signalling pathway is unable to drive PLGF release in the presence of CRHR1 antagonist, NBI27914. A trend towards increase in PLGF release is seen in the presence of CRHR-2 antagonist, Anti-Sauvagine 30. Thus, implying forskolin increases PLGF release in BEWO cells via CRHR1. A trend towards decrease in PLGF release is seen, possibly mediated via CRHR1 when CRH is added to syncytialised BEWO cells.

6.1.45 The regulation of release of PLGF by CRH via CRHR1 in syncytialised BEWO cells under high and low oxygen tension

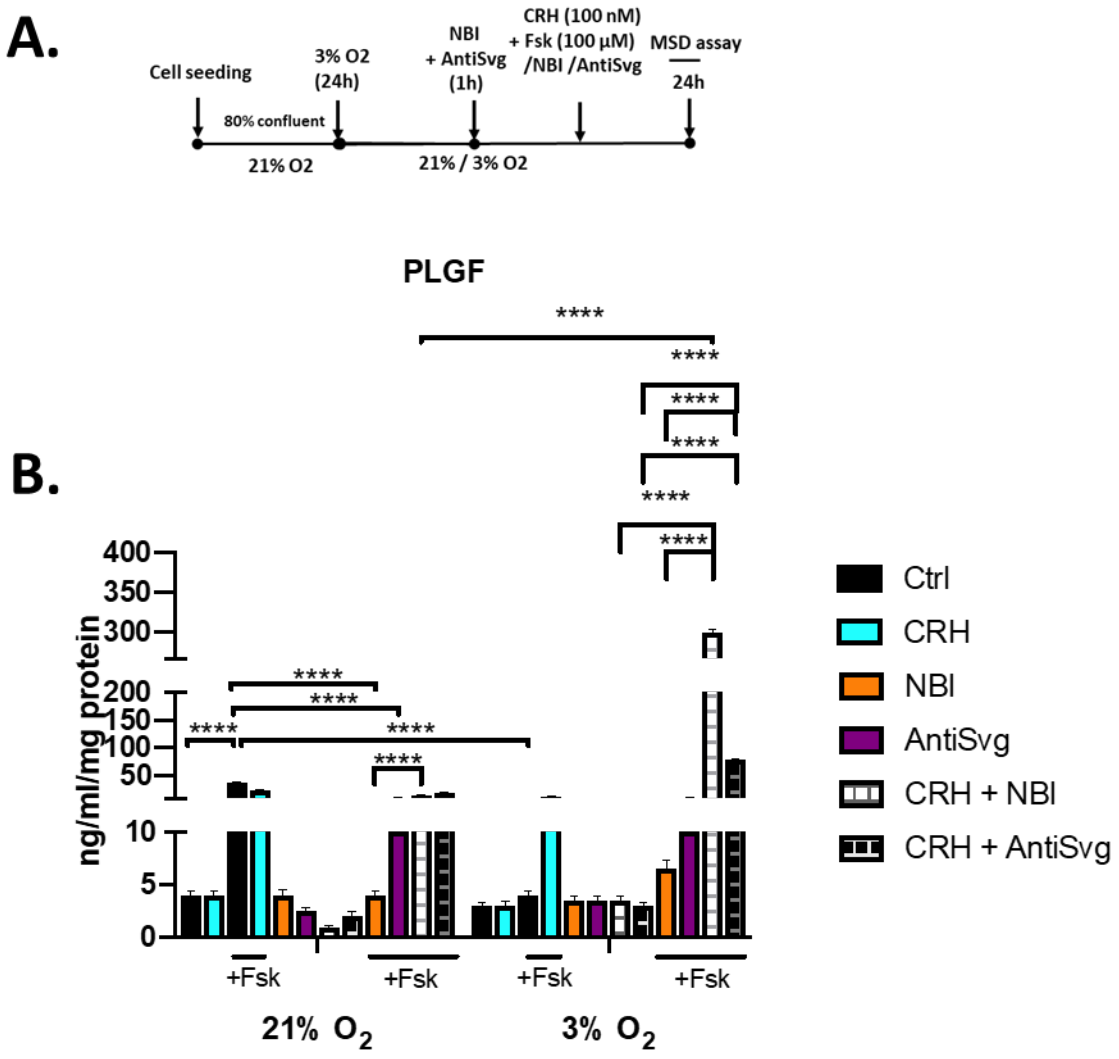


Figure 143

*Under low oxygen tension, forskolin does not increase PLGF release. CRHR2 prevents forskolin from increasing PLGF release. CRH increases PLGF release in syncytialised BEWO cells via CRHR1. A. Experiment protocol, where after 80% confluency, fresh media was added and cells were exposed to 3% O2 for 24h. Cells were pretreated with CRHR1 /2 antagonists, NBI 27914 or anti-sauvagine-30 for 1h. The cells were further treated with 100nM CRH along with 100um forskolin for 24h and supernatants were collected and MSD ELISA assay was done on PLGF protein. B. Under low oxygen tension, cells incubated with 100um forskolin for 24h showed no effect on PLGF release. Further combined treatment with NBI 27914 showed no increase in PLGF release. Cells treated with forskolin and antisauvagine – 30 showed an increase in PLGF release. Cells treated with CRH and forskolin showed an increase in PLGF release. Further combined treatment with NBI showed a significant increase in PLGF release. Cells treated with CRH, forskolin and anti-sauvagine-30 showed only a trend towards an increase in PLGF release. **** $p<0.0001$; n=3; CRH : corticotrophin releasing hormone. Data expressed as ng/ml normalised against mg protein. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

Table 58

PLGF release in BEWO	21% O2				3% O2			
	Ctrl	CRH	Fsk	CRH + Fsk	Ctrl	CRH	Fsk	CRH + Fsk
- NBI /AntiSvg-30	NS	NS	↑	NS	NS	NS	NS	↑
+ NBI	NS	NS	NS	↑	NS	NS	↑	↑
+ AntiSvg-30	NS	NS	↑	↑	NS	NS	↑	↑

The receptors involved in the regulation of the release of PLGF by CRH were investigated in syncytialised BEWO cells under low oxygen tension. BEWO cells under low oxygen tension were investigated by simultaneously incubating cells with 100nM CRH and 100µM forskolin for 24h. Some cells were preincubated for 1 hour with, CRHR-1 and 2 antagonists, NBI-27914 and Anti-Sauvagine 30 respectively and PLGF release was analysed using ELISA. Forskolin does not increase PLGF release and this may be mediated by CRHR2. PLGF release by cAMP signalling pathway was increased by 20 fold in the presence of CRHR2 antagonist, anti-Sauvagine-30. No effect is seen with CRHR1 antagonist, NBI 27914. Furthermore, when syncytialised BEWO cells were incubated with CRH, in the presence of CRHR1 antagonist, NBI -27914, PLGF release is increased upto 240 fold. Thus, the increase in PLGF release seen, when CRH is added to syncytialised BEWO cells, is possibly mediated via CRHR1. No effect is seen with CRHR2 antagonist, Anti-Sauvagine 30.

6.1.46 Expression of VEGF in syncytialised BEWO cells under high oxygen tension

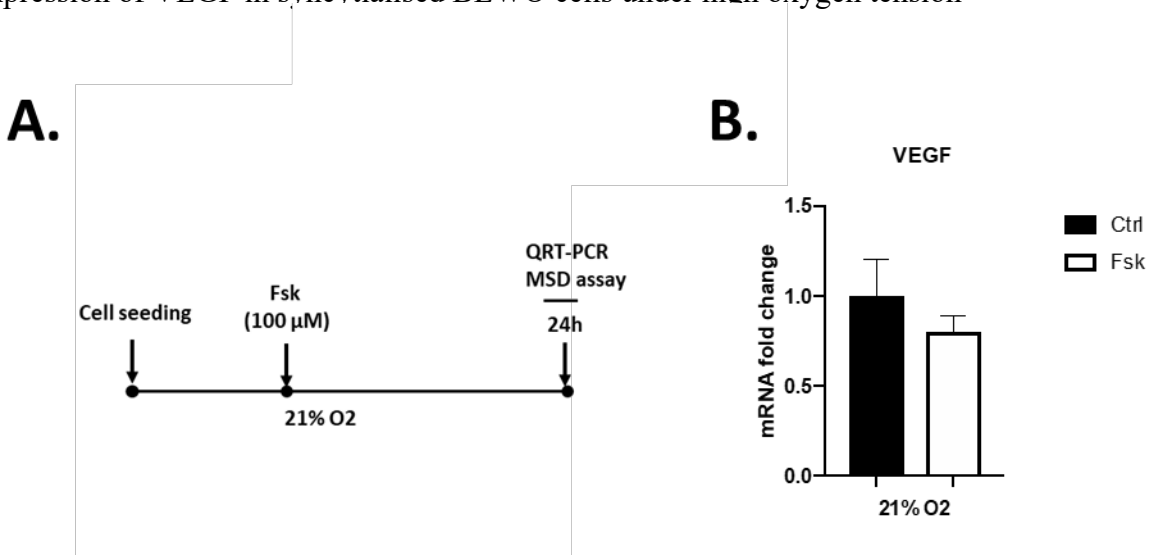


Figure 144

Under high oxygen tension, cAMP/PKA signalling pathway has no effect on VEGF mRNA expression from BEWO cells. 1. Experiment protocol. 2.(A) QRT-PCR analysis showed that the expression of VEGF MRNA remained unaffected in BEWO cells treated with 100um forskolin for 24h at 21% O2. n=3; Fsk: forskolin. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.

The expression of VEGF messenger RNA (mRNA) was analysed using real-time PCR in BEWO cells. To verify the activation of the cAMP-PKA pathway, BEWO cells were treated with 100 µM forskolin (a strong activator of adenylate cyclase) for 24h. Under high oxygen tension (21%O2), forskolin did not regulate VEGF mRNA expression compared to control.

6.1.47 Expression of VEGF in syncytialised BEWO cells under high and low oxygen tension

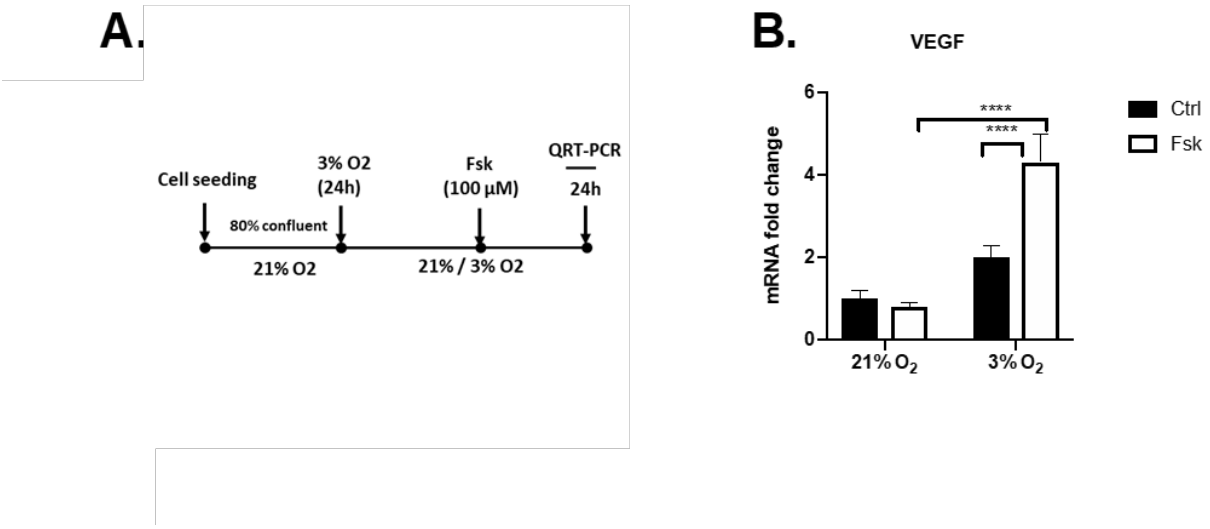


Figure 145

*Low oxygen tension increases VEGF mRNA expression in syncytialised BEWO cells.1. Experiment protocol; 2.(A) QRT-PCR analysis showed that the expression of VEGF MRNA was significantly upregulated in BEWO cells treated with 100Um forskolin for 24h at 3% O2. **** p<0.0001; n=3; Fsk: forskolin. Data expressed as (A) fold change of gene expression, relative to control 24h, after normalising against B-actin and (B) ng/ml normalised against mg protein. Data were analysed using a two-way ANOVA with Tukeys mutiple comparison test.*

The expression of VEGF messenger RNA (mRNA) was analysed using real-time PCR in BEWO cells. To verify the activation of the cAMP-PKA pathway, BEWO cells were treated with 100 µM forskolin (a strong activator of adenylate cyclase) for 24h. qRT - PCR analysis of BEWO cells undergoing forskolin induced syncytialization showed that VEGF mRNA expression is upregulated by 2 fold in BEWO cells compared to control under low oxygen tension (3%O2).

6.1.48 The regulation of VEGF by CRH in BEWO cells under high oxygen tension

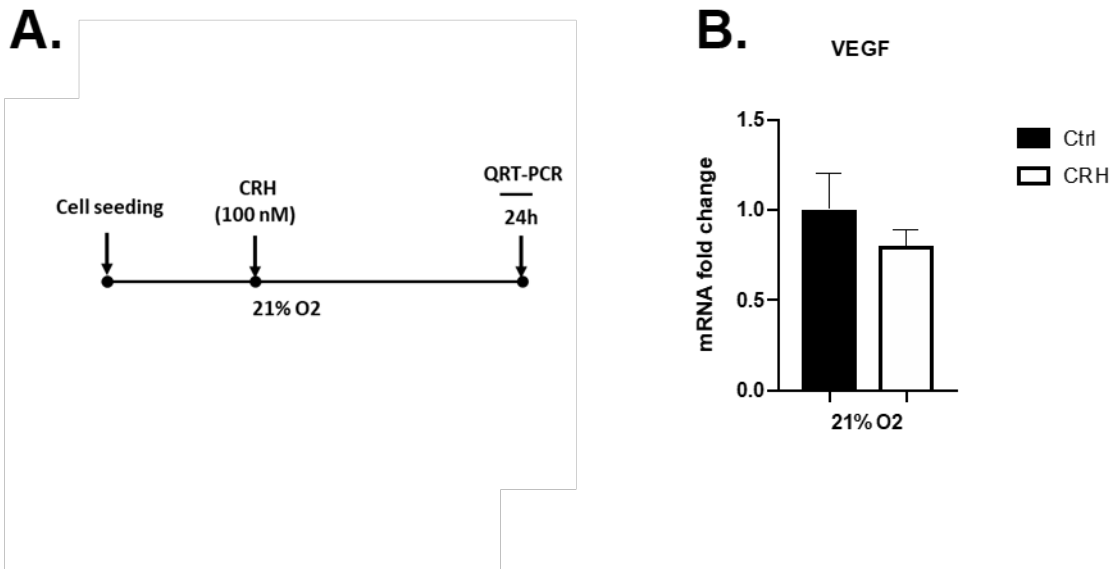


Figure 146

CRH has no effect on VEGF mRNA expression in BEWO cells under high oxygen tension. A) Experiment protocol; B) QRT-PCR analysis showed that the expression of VEGF MRNA remained unaffected in BEWO cells treated with 100nm CRH for 24h at 21% O2. n=3; CRH: corticotrophin releasing hormone. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.

The regulation of mRNA expression of VEGF by CRH was investigated in BEWO cells by incubating cells with 100nM CRH under high oxygen tension and VEGF mRNA was analysed using QRT-PCR. CRH had no effect on VEGF mRNA expression in BEWO cells under high oxygen tension (21% O2).

6.1.49 The regulation of VEGF by CRH in BEWO cells under low oxygen tension

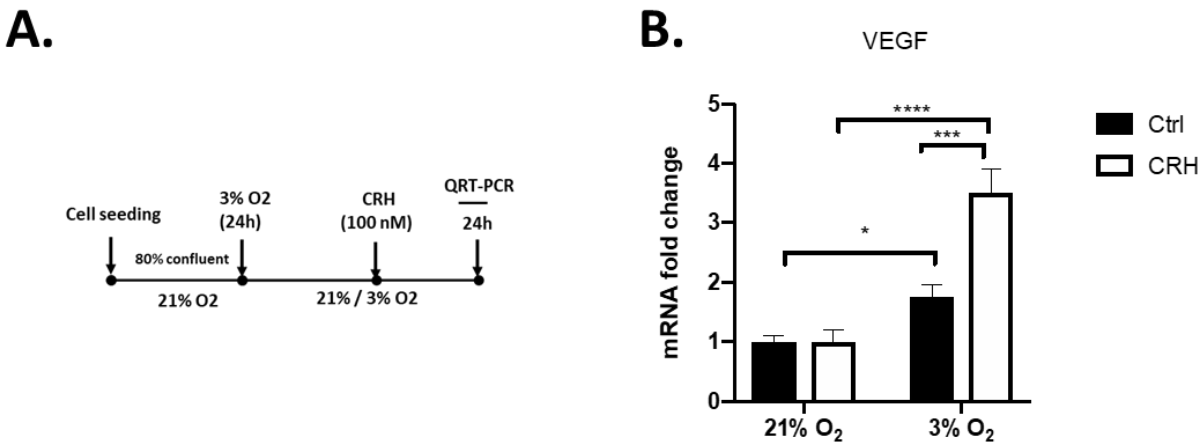


Figure 147

CRH potentiates the actions of hypoxia on VEGF mRNA expression.

(A) Experiment protocol; (B) QRT-PCR analysis showed that the expression of VEGF MRNA was significantly upregulated in BEWO cells exposed to 3% O2 for 24h. BEWO cells further treated with 100NM CRH showed that CRH further potentiated the actions of hypoxia on the expression of VEGF MRNA. p= 0.033, ** .002 ;*** .0002 ; **** p<0.0001; n=3; CRH : corticotrophin releasing hormone. Data expressed as (A) fold change of gene expression, relative to control 24h, after normalising against B-actin and (B) ng/ml normalised against mg protein. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

The regulation of expression of VEGF by CRH was investigated in BEWO cells under low oxygen tension by incubating cells with 100nM CRH and VEGF mRNA was analysed using QRT-PCR. A trend towards increase in VEGF mRNA was seen under low oxygen tension and CRH potentiated VEGF mRNA expression by 2 fold.

6.1.50 The regulation of VEGF by CRH in syncytialised BEWO cells under high oxygen tension

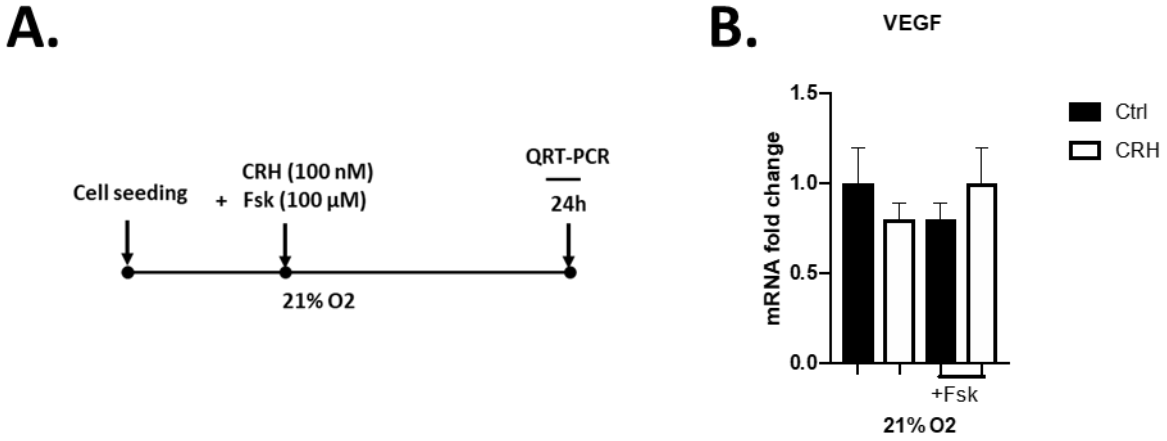


Figure 148

CRH has no effect on VEGF mRNA expression in syncytialised BEWO cells under high oxygen tension. (A) Experiment protocol; 2. (B) QRT-PCR analysis showed that the expression of VEGF MRNA was unaffected in BEWO cells treated with 100nm CRH and 100um forskolin, exposed at 21% O2 for 24h. n=3; Fsk: forskolin; CRH: corticotrophin releasing hormone. Data expressed as (A) fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a one-way ANOVA with Tukey's multiple comparison test.

The regulation of VEGF mRNA by CRH in syncytialised BEWO cells under high oxygen tension was investigated. VEGF mRNA expression remained unaffected in the presence of 100nM CRH in syncytialised BEWO cells.

The regulation of VEGF by CRH in syncytialised BEWO cells under low oxygen tension

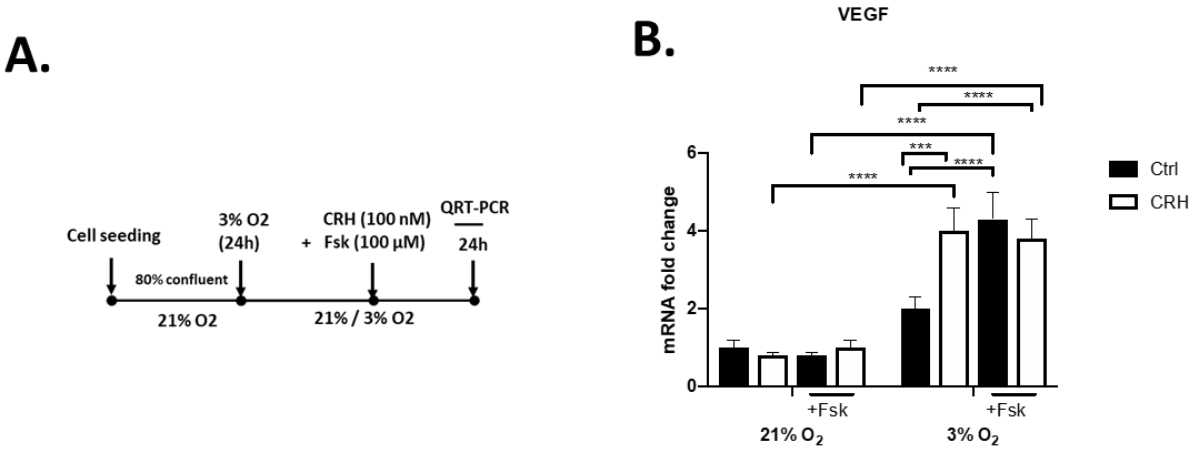


Figure 149

*Under low oxygen tension, CRH upregulates VEGF mRNA in BEWO cells but not in syncytialised BEWO cells.(A) Experiment protocol; (B) QRT-PCR analysis showed that the expression of VEGF MRNA was significantly upregulated in BEWO cells treated with 100NM CRH exposed at 3% O2 for 24h. Furthermore, some cells incubated with 100NM CRH along with 100um forskolin showed VEGF MRNA expression remained unaffected. ***p< .0002 ; **** p<0.0001; n=3; CRH : corticotrophin releasing hormone; fsk: forskolin. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

Table 59

BEWO	21% O2				3% O2			
	Ctrl	CRH	Fsk	CRH + Fsk	Ctrl	CRH	Fsk	CRH + Fsk
PLGF mRNA	NS	NS	↑	NS	↑	↑	↑	↓
VEGF mRNA	NS	NS	NS	NS	NS	↑	↑	NS

The regulation of VEGF mRNA by CRH in syncytialised BEWO cells under low oxygen tension was investigated. Under low oxygen tension, both forskolin (100 µM) and CRH upregulated VEGF mRNA by 2 fold. VEGF MRNA upregulation by forskolin was not affected in the presence of 100nM CRH.

Discussion

Syncytiotrophoblasts, which are in direct contact with the maternal blood, are the primary site of production and secretion of proangiogenic and antiangiogenic factors like vascular endothelial growth factor A (VEGF-A), placental growth factor (PlGF), and the soluble form of their common receptor SFLT-1. These factors simultaneously regulate fetal blood vessel formation in the placenta as well as maternal vascular function during the course of pregnancy.

BEWO is a good cellular model to study PLGF release, in comparison to other HUVEC, HTR8 and HMEC-1 models. Other molecules such as SFLT-1 and VEGF were also detected, but secreted to a lesser extent compared to HUVEC and HTR8 model. PLGF was endogenously secreted by BEWO cells in a time dependent manner, and the pattern of secretion reversed on exposure to low oxygen tension.

Fusion and differentiation of trophoblasts occur through unknown mechanisms in vivo, but this process can be mimicked by treatment with cAMP or forskolin in vitro. Cyclic 3',5'-adenosine monophosphate (cAMP) is a critical second messenger for human trophoblasts and regulates the expression of numerous genes. It is known to stimulate *in vitro* fusion and differentiation of BeWo choriocarcinoma cells, which acquire the characteristics of syncytiotrophoblasts. BEWO choriocarcinoma cells can be induced to fuse under these conditions, which is associated with a corresponding upregulation of syncytin and hCG production. The cAMP-PKA-CREB pathway has been implicated in several networks of human placental gene activation, including those involved in endocrine differentiation of trophoblasts. While the pathways are not fully elucidated, cAMP, through PKA, upregulates the expression and the transcriptional activity of the placenta-specific transcription factor, glial cells missing 1 (GCM1),[10] which binds to the syncytin gene promoter and upregulates its transcription.[11] Several other trophoblast genes are also upregulated by cAMP, including those encoding the α and β subunits of human chorionic gonadotropin (hCG) and aromatase (hCYP19).[12,13] These genes promote hCG and estrogen production. Hormones and small

peptides are also important in the regulation of the vascular function. Estrogens and progesterone modulate vascular tone during pregnancy and mediate aspects of maternal cardiovascular adaptation[2]; however in the setting of menopausal hormone therapy, these sex steroids are also linked to risks of venous thromboembolism.[3,4] Given the important role of cAMP in the regulation of trophoblast differentiation, it was postulated that it might also be involved in placental angiogenesis.As expected, ELISA and QRT-PCR analysis of BEWO cells undergoing forskolin-induced syncytialization showed that the β--HCG secretion was enhanced and the angiogenic growth factor PlGF gene was upregulated and protein secretion was increased.[19] Under low oxygen tension, fusion of BEWO cells were associated with an impairment of syncytin and β -HCG production. Hypoxia increases progesterone, estradiol and cortisol release. β –HCG and Progesterone release are enhanced in syncytialised bewo cells which is further impaired under low oxygen tension. In bewo cells, Low oxygen tension increases PLGF MRNA expression but not release. In syncytialised BEWO cells, low oxygen tension increased PLGF MRNA expression but reduces PLGF release. Low oxygen tension may affect the post transcriptional modification of PLGF. Thus indicating that PKA may be a potent regulator of *PlGF* gene expression and protein secretion in trophoblasts. This needs to be further confirmed with PKA inhibitor.

As observed in studies on placental villous explants and in choriocarcinoma cell lines https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3343062/#bibr19-1933719110389337,
PlGF gene was upregulated by cAMP. Vascular endothelial growth factor production was not affected by cAMP, this latter result might explain why VEGF was not cited as upregulated significantly during syncytialization in the cDNA microarray studies of Kudo et al.[19] . Another group also investigated VEGF protein secretion and the effect of forskolin was shown to be subtle https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3343062/#bibr19-1933719110389337. Similar to PLGF gene, a trend towards an increase in VEGF MRNA expression by low oxygen tension in bewo cells. Low oxygen tension increased VEGF MRNA expression in syncytialised BEWO cells. Further studies on VEGF and PLGF activation in syncytialised BEWO cells are necessary.

Circulating levels of VEGF-A and PlGF proteins can be detected in the maternal serum as early as 8 weeks' gestation. Longitudinal studies measuring PlGF in the serum of pregnant women by ELISA show that free PlGF continues to increase during the course of pregnancy but starts to decline around 29 to 32 weeks' gestation. The control of its secretion remains unexplained.[15,16]

Placental angiogenesis, which can be detected in the human as early as 1 week postimplantation, continues until term. Several factors regulating placental angiogenesis are VEGF-A, PlGF, sVEGFR1, and soluble Endoglin (sEng). Alteration in the expression of these factors is associated with abnormal vascularization of the placental bed that is a hallmark of the pregnancy-related diseases, PE and intrauterine growth restriction (IUGR). In the serum of women with PE, total VEGF levels are reported to be increased[14] while free PlGF levels are decreased[15,16]; at the same time, the antiangiogenic factors sVEGFR1 and sEng are both increased.[17]

The biosynthesis of cAMP classically is stimulated by binding of a ligand to G-protein-coupled membrane receptors (GPCRs), with subsequent activation of adenylate cyclase, the regulatory subunit of protein kinase A (PKA), and ultimately phosphorylation of the transcription factor cAMP-responsive element-binding protein (CREB). Phosphorylated CREB homodimerizes and binds to cAMP-responsive elements (CREs) located in the promoters of its target genes, leading to the initiation of their transcription.

The activation of the cAMP/PKA-signaling pathway, mediated by membrane adenylate cyclase and Gα stimulatory proteins, suggests that 1 or more GPCR is responsible for our observation. G-protein-coupled membrane receptors represent the largest class of cell surface receptors, comprising about 200 GPCRs with known ligands and 80 orphan GPCRs. Several of these are expressed in the placenta and are under active investigation presently.

Interesting candidate ligands that could increase intracellular cAMP via GPCRs include calcitonin gene-related peptide (CGRP) and adrenomedullin (AM). Both molecules are expressed in the placenta and are known to have vascular effects.[36,37] The 2 ligands share a common membrane receptor, the calcitonin receptor-like receptor (CRLR), which is associated with specific receptor activity modifying proteins (RAMPs) and the G-protein complex.

We previously observed low PlGF levels in the circulation of women destined to develop PE and IUGR[15] and suggested that abnormal production of angiogenic factors like PlGF might be characteristic of pregnancy-related diseases.[1] Preeclampsia is characterized by hypertension and proteinuria and is associated with low levels of CGRP and CRLR mRNA in placental samples.[38–41] However, some reports indicate that AM mRNA and protein may be increased in the placentae and fetoplacental circulation of women with PE and IUGR.[42–44]

CRH increases PLGF MRNA expression under high oxygen tension. CRH further potentiates the actions of hypoxia on PLGF MRNA expression but not reflected at protein release level. CRH does not regulate forskolin induced PLGF MRNA expression and protein secretion under high oxygen tension in BEWO cells. Under low oxygen tension, CRH reduces PLGF MRNA in syncytialised BEWO cells. Under high oxygen tension, CRH reduces PLGF release at 6h but has no effect at other time points. Under low oxygen tension, CRH does not regulate PLGF release in BEWO cells. Under high but not low oxygen tension, blocking CRHR1 reduces CRH mediated PLGF release. Under high oxygen tension,CAMP signalling pathway increases PLGF release via CRHR1. Under low oxygen tension, forskolin does not increase PLGF release. CRHR2 prevents forskolin from increasing PLGF release. CRH increases PLGF release in syncytialised BEWO cells via CRHR1. CRH has no effect on VEGF MRNA expression in BEWO cells under high oxygen tension. CRH potentiates the actions of hypoxia on VEGF MRNA expression. CRH has no effect on VEGF MRNA expression in syncytialised BEWO cells under high oxygen tension. Under low oxygen tension, CRH upregulates VEGF MRNA in BEWO cells but not in syncytialised BEWO cells. A more thorough comprehension of how PlGF is regulated during normal pregnancy is an important prerequisite to understanding its abnormal secretion, as observed in the serum of women developing pregnancy-related diseases like PE and IUGR.

Table 60

Summary of PLGF regulation by CRH in syncytialised BEWO cells under high and low oxygen tension

PLGF	21% O2								3%O2							
	mRNA				Release				mRNA				Release			
	-Fsk		+Fsk		-Fsk		+Fsk		-Fsk		+Fsk		-Fsk		+Fsk	
	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH	Ctrl	CRH
- NBI/AntiSvg		↑	↑	NS	↑	↓	↑	NS	↑	↑	↑	↓	↓	NS	↓	↑
+ NBI						↓		↑							↑	↑
+AntiSvg							↑	↑							↑	↑

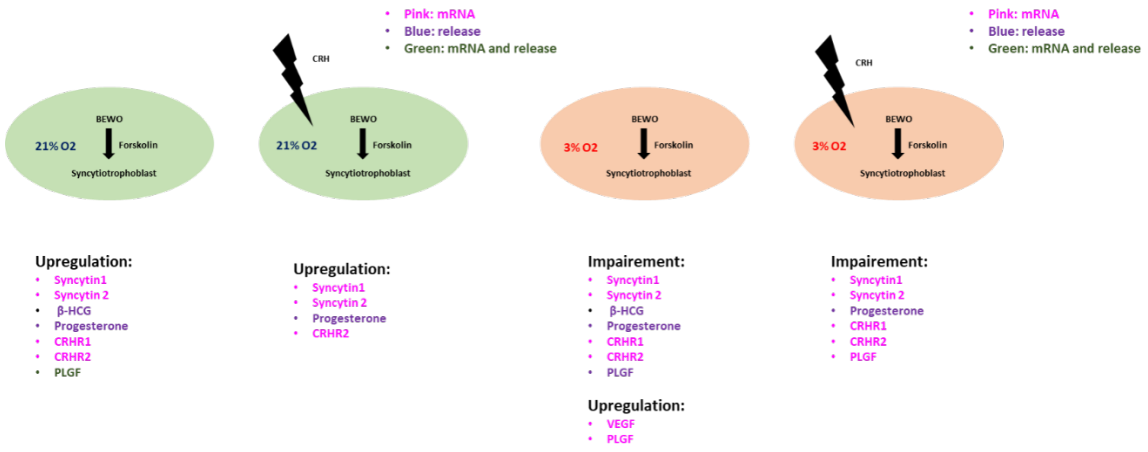


Figure 150

Pictorial representation of regulation of angiogenic growth factors and fusion machinery by CRH in BEWO cells under high and low oxygen tension.

6.1.51 Role of 11 β-HSD2 on eNOS, AKT and BCL-2 protein expression in BEWO differentiation

The dynamics and temporal characteristics of 11 β-HSD2 inhibition are not well understood. In order to understand a possible role for 11 β-HSD2 in regulation of cellular processes such as proliferation and apoptosis in villous cytotrophoblast differentiation, protein expression of eNOS, AKT, BCL-2 was assessed using western blot technique.

Positive control: VEGF was used as a positive control to produce the expected effect with the assay or experimental set up. VEGF significantly upregulated AKT compared to control cells, which is in agreement with previous studies (Gerber, McMurtrey et al. 1998).

6.1.52 Effect of cAMP signal transduction on regulation of eNOS protein expression and the role of 11 β-HSD2 in this process

11 β-HSD2 activity can be modulated by stimulation of cAMP pathway(Pasquarette, Stewart et al. 1996). The functional role of eNOS in trophoblasts, specifically during differentiation remains less well characterized. The aim of this experiment was to assess eNOS protein expression in undifferentiated and differentiated (forskolin) BEWO cells as well as to investigate the influence of 11 β-HSD2 on eNOS expression during trophoblast differentiation. Previous studies showed that inhibition of eNOS resulted in increased 11 β-HSD2 expression in BEWO(A. Hein 2012). In this experiment, carbenoxolone had no significant direct effects on eNOS protein expression at 24 hour or 48-hour time period. A significant downregulation of eNOS protein expression by forskolin was observed at 48 hours, but not at 24 hours. Forskolin induced eNOS downregulation was further suppressed in the presence of carbenoxolone both at 24 hours and 48 hours. This implies that under basal conditions, 11 β-HSD2 has no effect on eNOS expression, and that a possible effect of 11 β-HSD2 is apparent only under conditions that induce eNOS expression. Forskolin inhibits eNOS and carbenoxolone accelerates the effect of forskolin on eNOS and may alter its rate of inhibition. Previous studies showed that forskolin induces 11 β-HSD2 mRNA and activity in cultured human placental and chorionic trophoblasts(Sun, Yang et al. 1998). Thus, there appears to be a delayed inhibitory effect of trophoblast differentiation on eNOS protein expression. cAMP has a delayed response at 48 hours on eNOS protein expression. Probably eNOS is essential for proliferation of cells, and that inhibition of proliferation may help to drive the

cells towards differentiation. cAMP may have potent, accelerated inhibitory effect on eNOS protein expression in absence of 11 β-HSD2. Besides, pretreatment with carbenoxolone for 1 hour was not sufficient as the actions of carbenoxolone appears to be be reversed on its removal. The presence of carbenoxolone along with forskolin for 24 hours or 48 hours appear to have potent effects compared to pretreatment with carbenoxolone for 1 hour, because their actions appear to be reversed on its removal. The presence of 11 β-HSD2 has a role in preventing the inhibitory effects of cAMP on eNOS expression. These results may overall reflect how trophoblast cells may respond to glucocorticoids (refer figure).

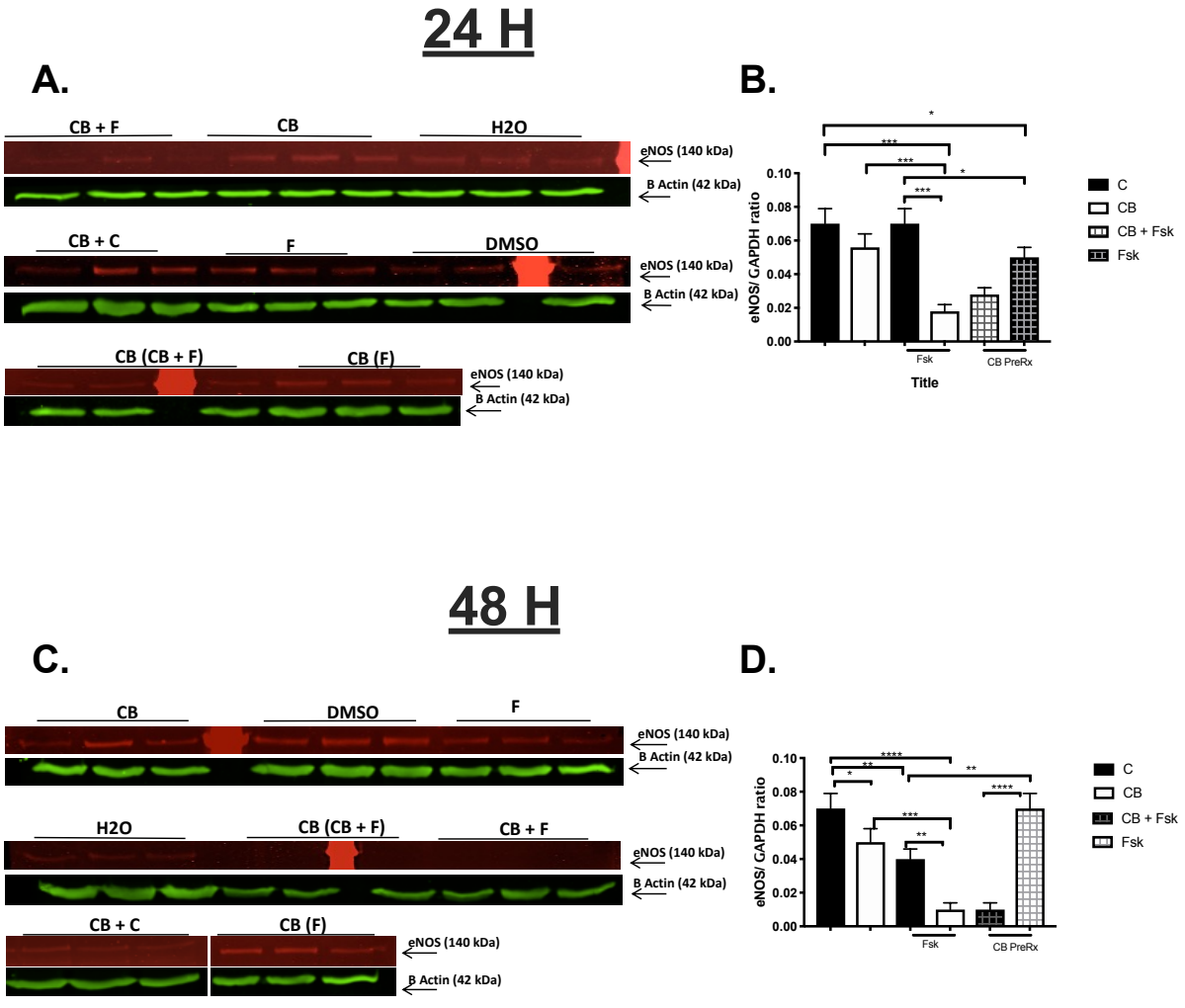


Figure 151

*Western blot analysis of effect of carbenoxolone and forskolin on eNOS protein expression in BEWO cells at (A) 24 hours, (C) 48 hours. Densitometry analysis was done was using eNOS/B actin ratio at (B) 24 hours and (D) 48 hours. Carbenoxolone inhibits forskolin induced eNOS downregulation at 24 hours and 48 hours. CB, carbenoxolone (100µM); F, forskolin (100µM); CB+F, carbenoxolone and forskolin; CB +C, pretreatment of carbenoxolone (100µM) for 1 hour which was followed by vehicle/ control for 24 hours or 48 hours; CB(CB+F), pretreatment of carbenoxolone (100µM) for 1 hour which was followed by combination of carbenoxolone and forskolin for 24 hours or 48 hours; CB (F), pretreatment of carbenoxolone (100µM) for 1 hour which was followed by forskolin for 24 hours or 48 hours. DMSO and H_20 were used as vehicles. eNOS (140kDa), β actin (42kDa). Overnight serum synchronization was done with 2% FBS. All treatments were in 2% FBS. All results are the mean (±SEM) /representative images of single experiment performed in triplicate(n=3). 25µg protein was loaded, some wells were loaded with 30µg protein. *p<0.05.*

Table 61

	ENOS		AKT		BCL2	
	24H	48H	24H	48H	24H	48H
Ctrl	NS	NS	NS	NS	NS	NS
CB	NS	↓	↓	↓	NS	NS
Fsk	NS	↓	NS	↓	↑	NS
CB + Fsk	↓	↓	↓	NS	↑	↑
CB (CB + Fsk)	NS	NS	NS	NS	NS	NS
CB (Fsk)	↓	↑	NS	↑	NS	NS

6.1.53 Effect of cAMP signal transduction on regulation of AKT protein expression and the role of 11 β-HSD2 in this process

The aim of this experiment was to evaluate the regulation of AKT by 11 β-HSD2 in cAMP mediated differentiation of BEWO cells. AKT regulates diverse cellular functions including growth, survival and proliferation. PI3K/AKT signaling pathway appears to function in parallel with the cAMP pathway and modulate endocrine differentiation of trophoblasts(Kent, Konno et al. 2010, Vatish, Tesfa et al. 2012). It has previously shown that in association with increased cAMP levels, simultaneous inhibition of PI3K/AKT pathway and reduction of intracellular calcium resulted in BEWO cell fusion(Vatish, Tesfa et al. 2012). In agreement to previous studies(Vatish, Tesfa et al. 2012), forskolin significantly reduced AKT protein expression at 48 hours. Carbenoxolone reduced AKT expression at 24 and 48 hours. Forskolin induced AKT downregulation was further suppressed in the presence of carbenoxolone. This

implies that forskolin itself is a potent inhibitor of AKT at 48 hours. Carbenoxolone accelerates the effect of forskolin on AKT and may alter its rate of inhibition. 11 β-HSD2 dampens the potential of forskolin actions on AKT expression in trophoblasts (refer figure). Previous studies showed knock out of 11 β-HSD2 using small interfering RNA (siRNA) causes inhibition of eNOS, AKT, β-HCG in BEWO. My preliminary data regarding carbenoxolone studies have been in agreement with siRNA based studies(A. Hein 2012). The limitation of this experiment was that only expression levels and not phosphorylation levels are being studied. Similar to eNOS, 11 β-HSD2 prevents the inhibitory effects of cAMP on AKT expression.

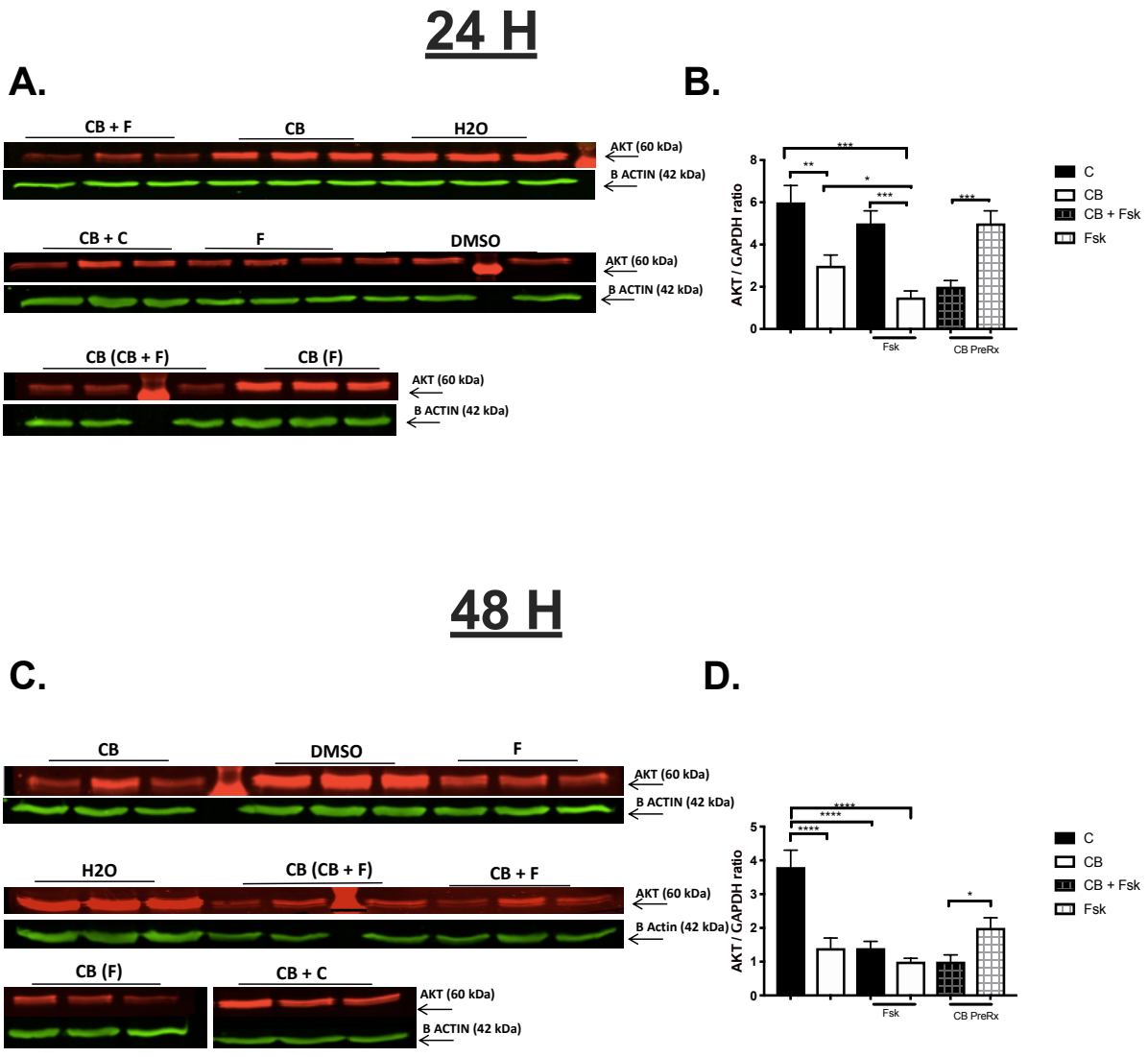


Figure 152

*Western blot analysis of effect of carbenoxolone and forskolin on AKT protein expression in BEWO cells at (A) 24 hours, (C) 48 hours. Densitometry analysis was done was using AKT/β actin ratio at (B) 24 hours and (D) 48 hours. Carbenoxolone inhibits AKT protein expression at both 24 hours and 48 hours. Forskolin inhibits AKT expression at 48 hours. Carbenoxolone inhibits forskolin induced AKT downregulation at 24 hours and 48 hours. CB, carbenoxolone (100μM); F, forskolin (100μM); CB+F, carbenoxolone and forskolin; CB +C, pretreatment of carbenoxolone (100μM) for 1 hour which was followed by vehicle or control for 24 hours or 48 hours; CB(CB+F), pretreatment of carbenoxolone (100μM) for 1 hour which was followed by combination of carbenoxolone and forskolin for 24 hours or 48 hours; CB (F), pretreatment of carbenoxolone (100μM) for 1 hour which was followed by forskolin for 24 hours or 48 hours. DMSO and H_20 were used as vehicles. AKT (60kDa), β actin (42kDa). Overnight serum synchronization was done with 2% FBS. All treatments were in 2% FBS. All results are the mean (±SEM) /representative images of single experiment performed in triplicate(n=3). 25μg protein was loaded, some wells were loaded with 30μg protein. *p<0.05.*

6.1.54 Effect of cAMP signal transduction on regulation of BEWO survival machinery and the role of 11 β-HSD2 in this process

My previous experiment showed inhibition of 11 β-HSD2 results in inhibition of AKT expression. AKT has been reported to increase expression of BCL-2, anti-apoptotic protein, so as to promote cell survival(Pugazhenthi, Nesterova et al. 2000). Syncytiotrophoblast differentiation is associated with alterations in the activity of apoptosis related proteins(Huppertz, Frank et al. 1998, Straszewski-Chavez, Abrahams et al. 2005). The aim of this experiment was to assess the regulation of BCL-2 by 11 β-HSD2 during intracellular fusion. Elevated BCL-2 protein levels were seen at 24 hours was seen in forskolin treated cells compared with vehicle treated controls. However, at 48 hours, reduced BCL-2 levels were observed but there was no statistical significance. This may imply that induction of trophoblast syncytialization leads the cells to enter into proapoptosis stage so as help them to differentiate it. BCL-2 protein expression is elevated at 24 hours, thus probably promoting the cells to proliferate so as to facilitate differentiation and thus antagonize apoptotic signals of proapoptotic proteins. Towards the completion of differentiation (48 hours), BCL-2 protein expression decreases. Also, the trend was reversed with cotreatment of BEWO with carbenoxolone and forskolin at both 24 hours and 48 hours. Carbenoxolone suppressed the forskolin induced BCL-2 levels at 24 hours; whereas at 48 hours, it augmented forskolin induced BCL-2 expression. This data suggests that BCL-2 levels may be seen elevated as a survival mechanism so as to resist cAMP mediated apoptosis at early stages but over the period of time, the resistance is lost. Carbenoxolone probably has no direct effects on BCL-2. BCL-2 is differentially regulated by 11 β-HSD2 compared to AKT and eNOS. 11 β-HSD2 controls apoptosis by regulating expression of BCL-2 proteins during differentiation. Inhibition of 11 β-HSD2 may enhance the sensitivity to apoptosis during BEWO differentiation.

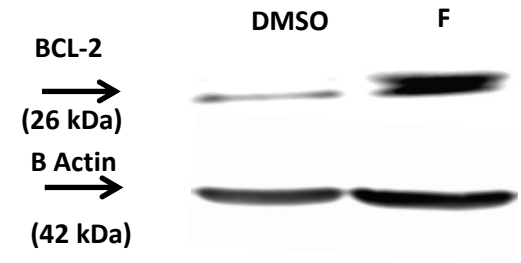


Figure 153

Western blot analysis (ECL) of effect of carbenoxolone and forskolin on BCL-2 protein expression in BEWO cells at (A) 24 hours, (C) 48 hours.

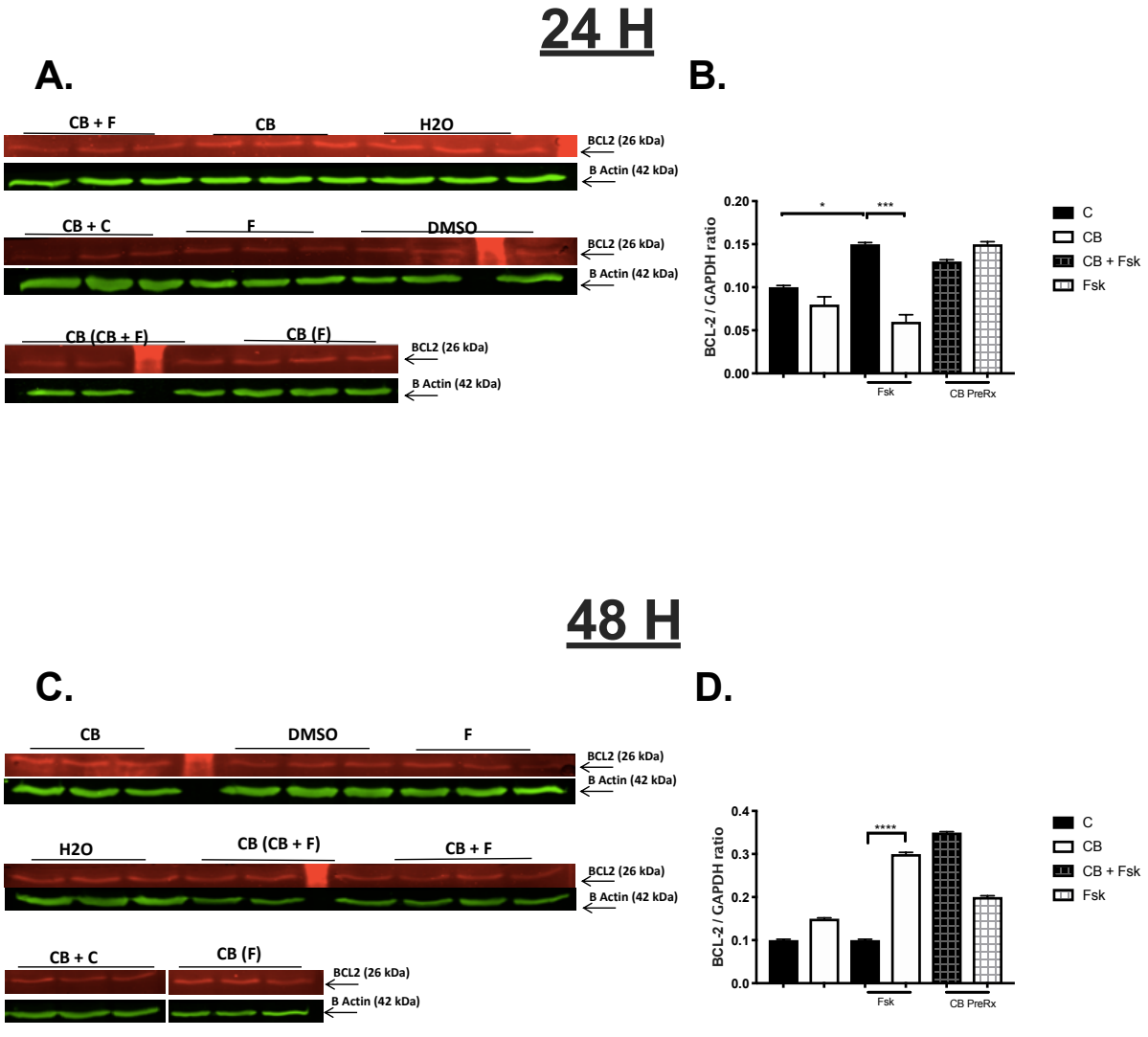


Figure 154

Western blot analysis of effect of carbenoxolone and forskolin on BCL-2 protein expression in BEWO cells at (A) 24 hours, (C) 48 hours. Densitometry analysis was done was using BCL-2/B actin ratio at (B) 24 hours and (D) 48 hours. Forskolin increases BCL-2 protein levels at 24 hours. Carbenoxolone downregulates forskolin induced BCL-2 protein expression at 24 hours and upregulates forskolin induced BCL-2 protein expression at 48 hours. CB, carbenoxolone (100μM); F, forskolin

*(100μM); CB+F, carbenoxolone and forskolin; CB +C, pre-treatment of carbenoxolone (100μM) for 1 hour which was followed by vehicle or control for 24 hours or 48 hours; CB(CB+F), pre-treatment of carbenoxolone (100μM) for 1 hour which was followed by combination of carbenoxolone and forskolin for 24 hours or 48 hours; CB (F), pre-treatment of carbenoxolone (100μM) for 1 hour which was followed by forskolin for 24 hours or 48 hours. DMSO and H20 were used as vehicles. BCL-2 (26kDa), β actin (42kDa). Overnight serum synchronization was done with 2% FBS. All treatments were in 2% FBS. 25μg protein was loaded, some wells were loaded with 30μg protein. All results are the mean (±SEM) /representative images of single experiment performed in triplicate(n=3). 25μg protein was loaded, some wells were loaded with 30μg protein. *p<0.05.*

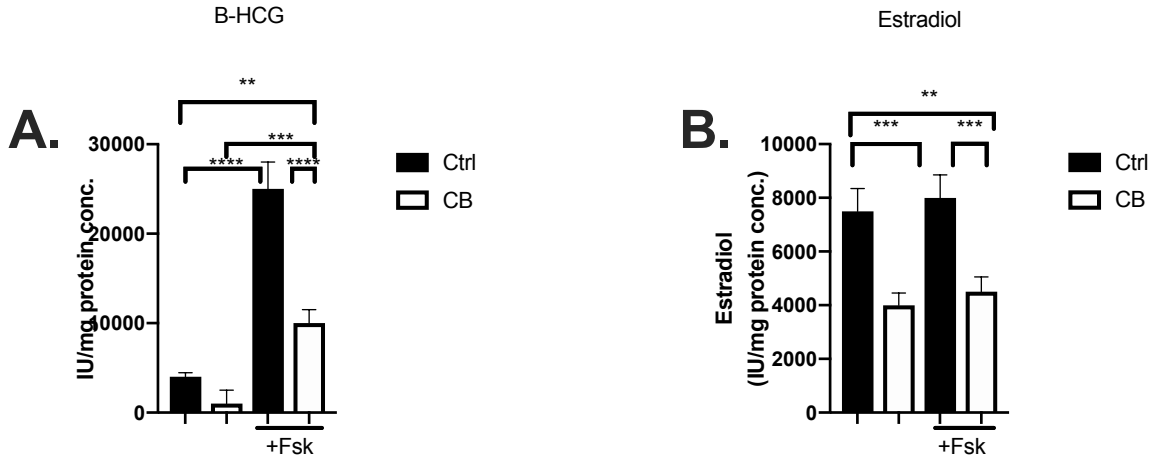


Figure 155

Table 62

	B – HCG	Estradiol
Ctrl	NS	NS
CB	NS	↓
Fsk	↑	NS
CB + Fsk	↓	↓

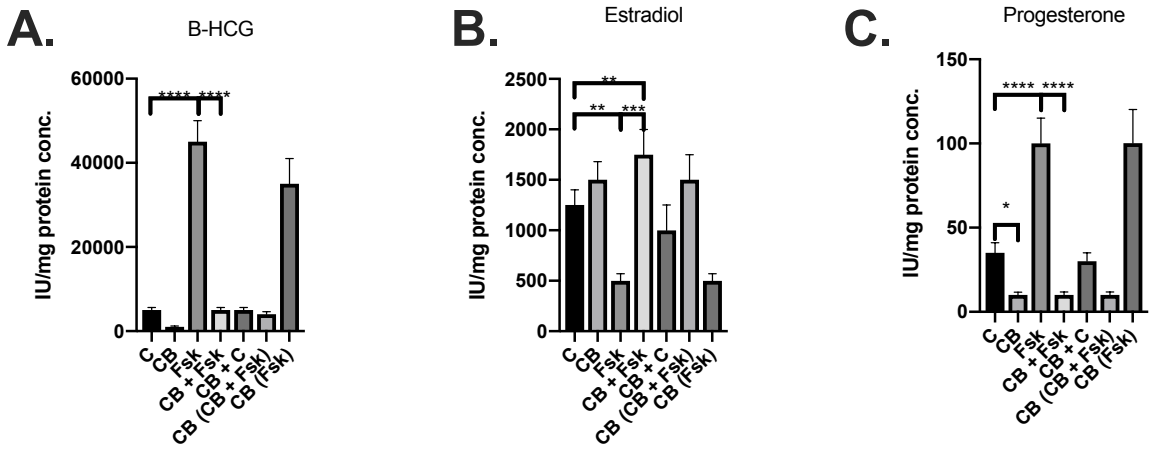


Figure 156

Table 63

BEWO 15’

	B – HCG	Estradiol	Progesterone
Ctrl	NS	NS	NS
CB	NS	NS	↓
Fsk	↑	↓	↑
CB + Fsk	↑	↓	↓
CB + C	NS	NS	NS
CB (CB + Fsk)	NS	NS	NS
CB (Fsk)	NS	NS	NS

6.2 HTR8

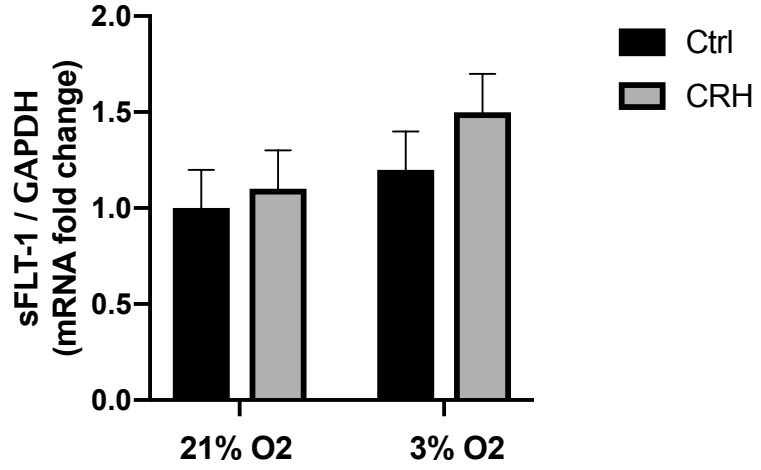


Figure 157

CRH has no effect on sFLT-1 mRNA expression under high and low oxygen tension in HTR8 / SVneo cells. QRT-PCR analysis showed that the expression of sFLT-1 mRNA were unaffected in BEWO cells treated with 100nM CRH, exposed at 21% O2 and 3% O2 for 24h. NS = non significant; n=3; CRH: corticotrophin releasing hormone. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.

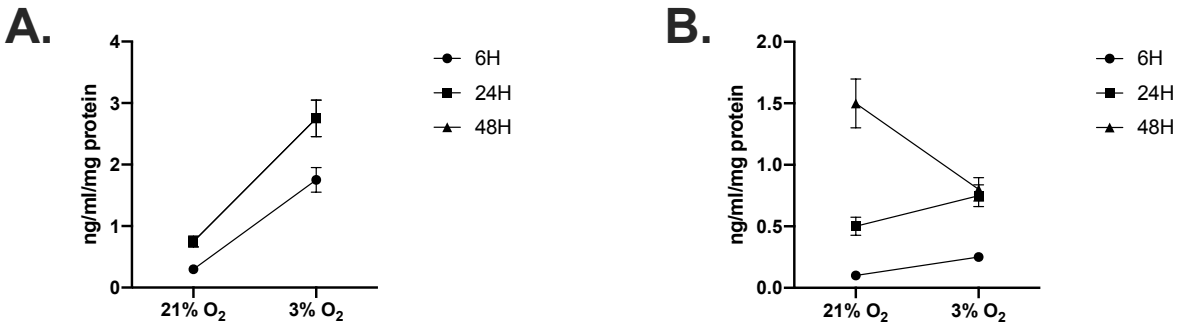


Figure 158

time dependent release of sFLT-1 and PLGF in HTR8 cells under low oxygen tension. Time dependent release of sFLT-1 (A) and PLGF (B) release in HTR8 /SVneo cellular models at 6h, 24h and 48h. Hypoxia increased basal sFLT-1 secretion in a time dependent manner upto 24h and reached a saturation at 48h. A time dependent increase in basal PLGF secretion was seen under high oxygen tension. Hypoxia had no effect on PLGF release and reached a saturation at 48h.

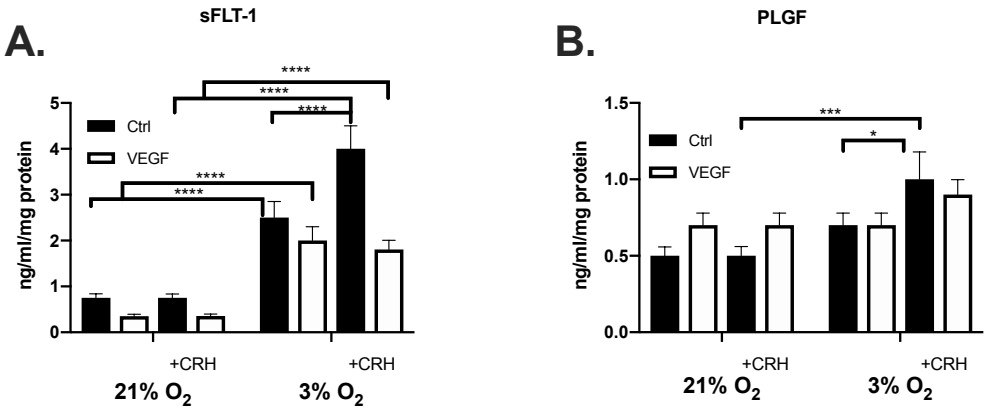


Figure 159

Hypoxia increased sFLT-1 protein release but not PLGF protein release. Under low oxygen tension, CRH increased sFLT-1 and PLGF release.

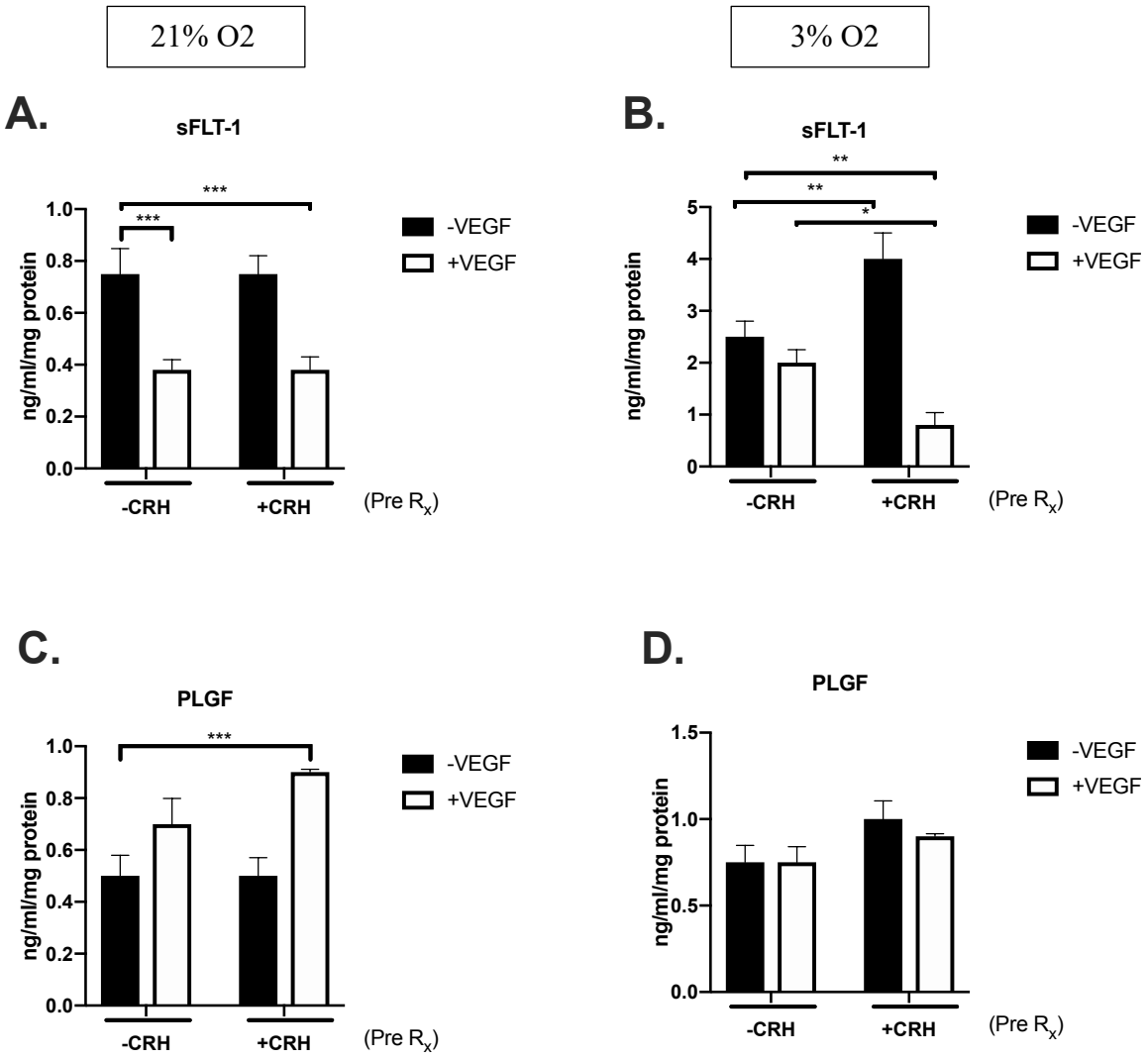


Figure 160

Priming cells with CRH for 6h had no effect on VEGF mediated regulation of sFLT-1 and PLGF release in 24h under high and low oxygen tension.

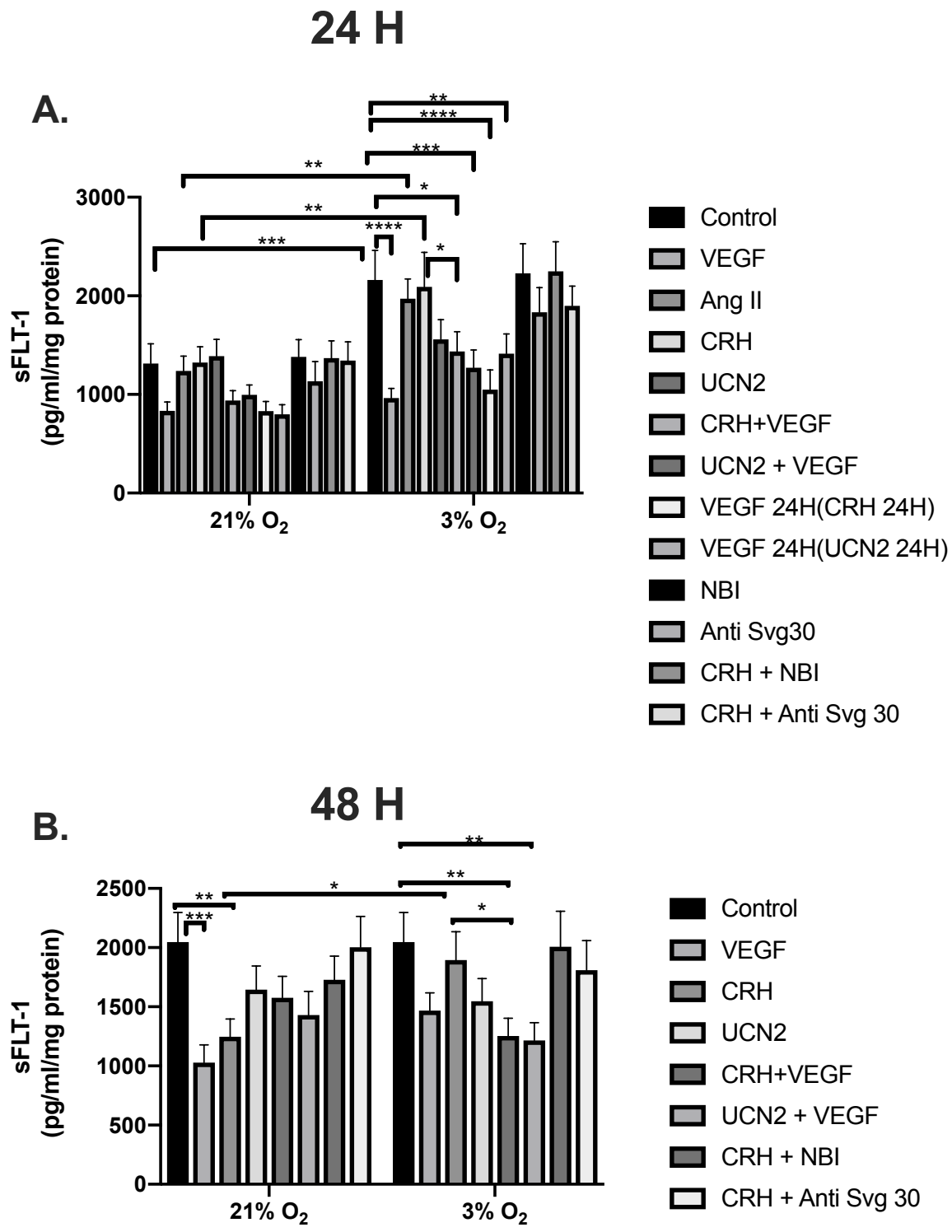


Figure 161

Hypoxia increases sFLT-1 protein secretion at 24h, which is lost at 48h. under high oxygen tension, VEGF reduces sFLT-1 protein secretion at 48 h but not at 24h. Under low oxygen tension, the pattern is reversed and VEGF reduces sFLT-1 protein secretion at 24h, which is lost at 48h.

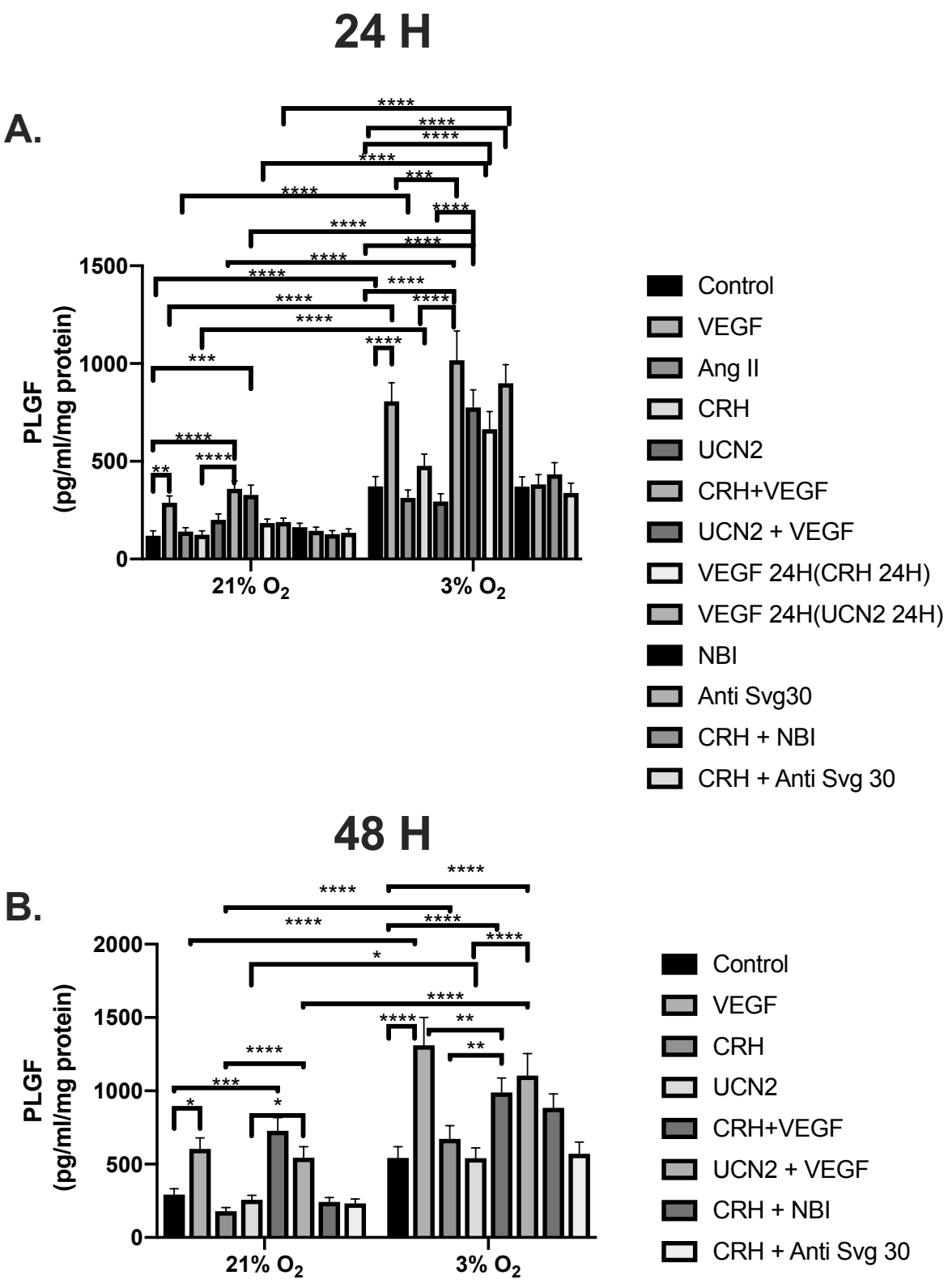


Figure 162

Under high oxygen tension, VEGF increases PLGF protein secretion in a time dependent manner upto 48h. Under low oxygen tension, VEGF increases PLGF protein secretion in a time dependent manner upto 48h. CRH reduces the ability of VEGF to induce PLGF secretion at 48h under low oxygen tension.

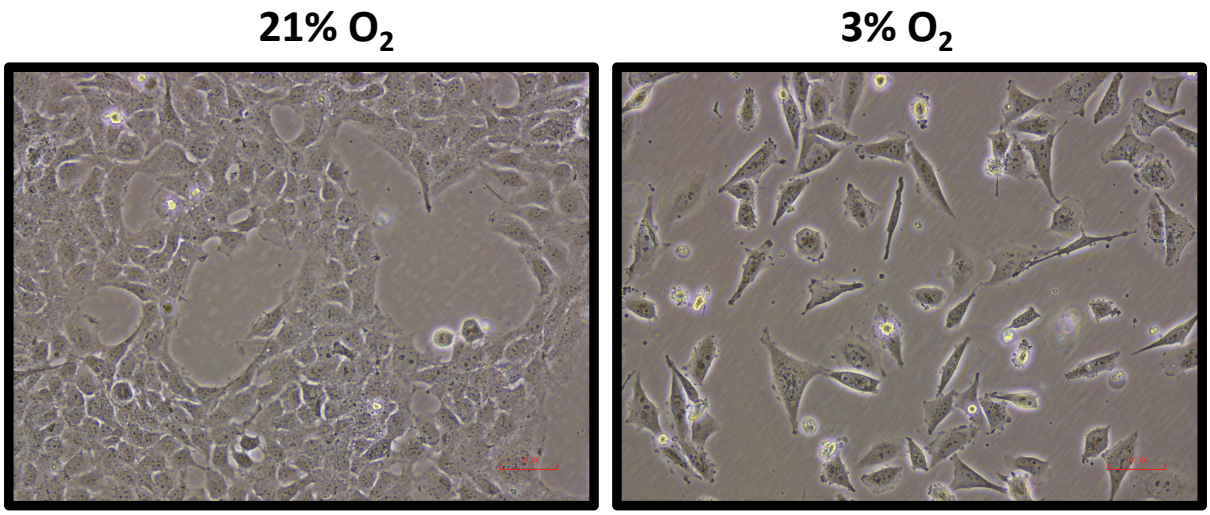


Figure 163

80% confluency of HTR8/SVneo cells under (A) high oxygen tension (B) low oxygen tension. Hypoxia changes the morphology and reduces the cell number of HTR8/SVneo cells.

The effect of conditioned media obtained from HUVEC cells on HTR8/SV neo cells under high and low oxygen tension

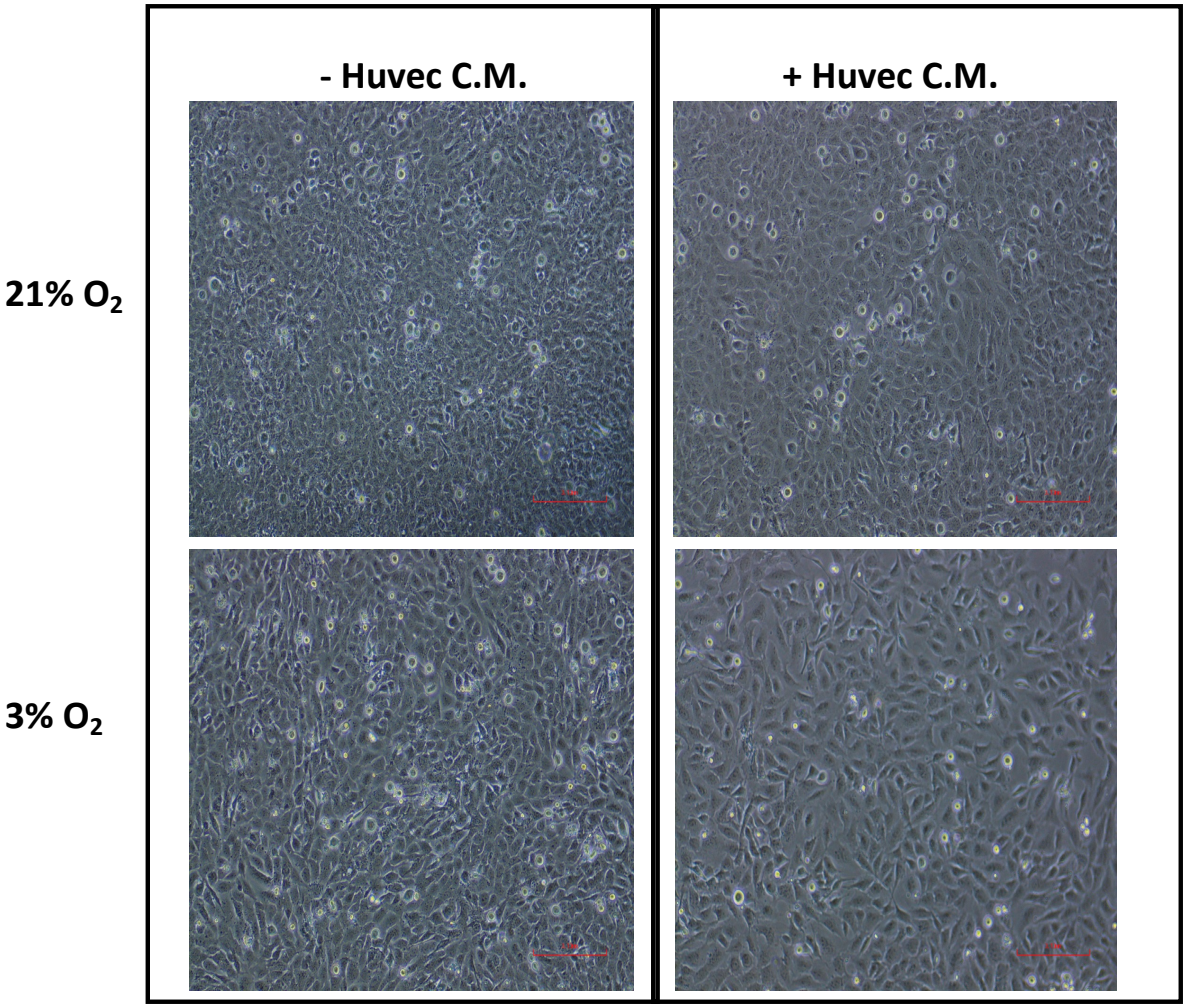


Figure 164

Conditioned media obtained from HUVEC cells has no effect on HTR8/SVneo cells under both high and low oxygen tension.

CRHR2 localization in HTR8/SVneo cells at different time points under high and low oxygen tension

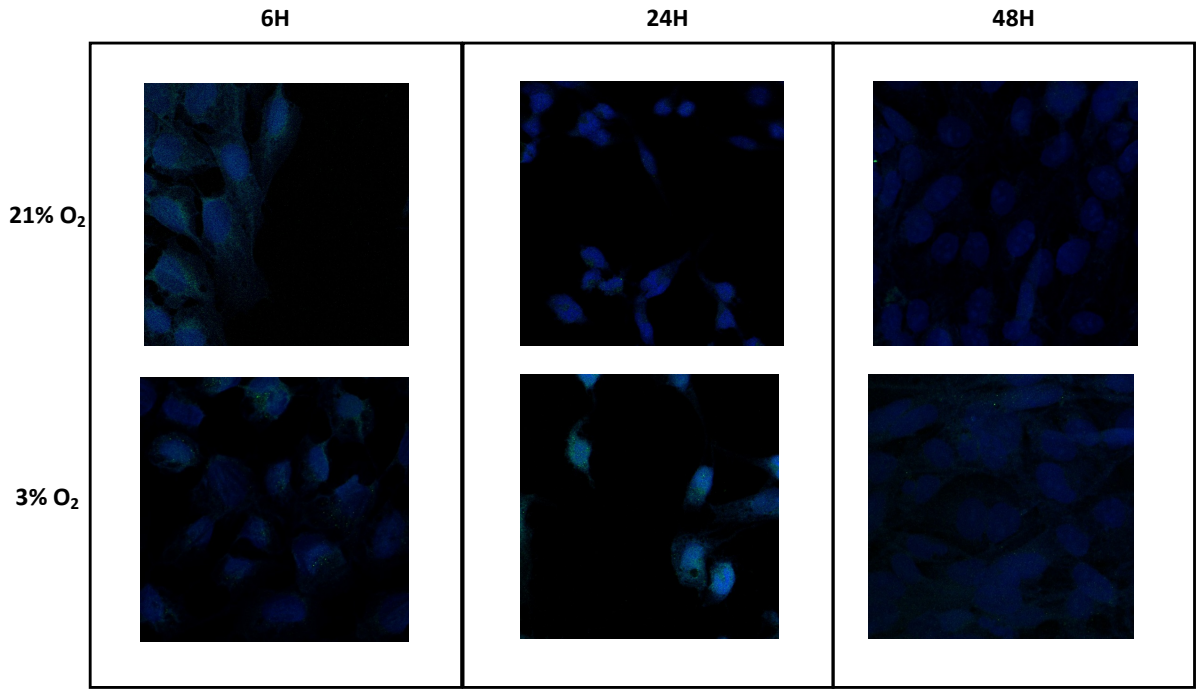


Figure 165

No CRHR2 localization was observed at 6h, 24h and 48h under high and low oxygen tension in HTR8/SVneo cells . CRH : corticotrophin releasing hormone; HUVEC C.M.: conditioned media obtained from HUVEC cell culture; DAPI: blue nuclei; CRHR2 : green Alexa fluor 488.

CRHR2 localization on adding HUVEC conditioned media on HTR8/SVneo cells under high and low oxygen tension at 6h.

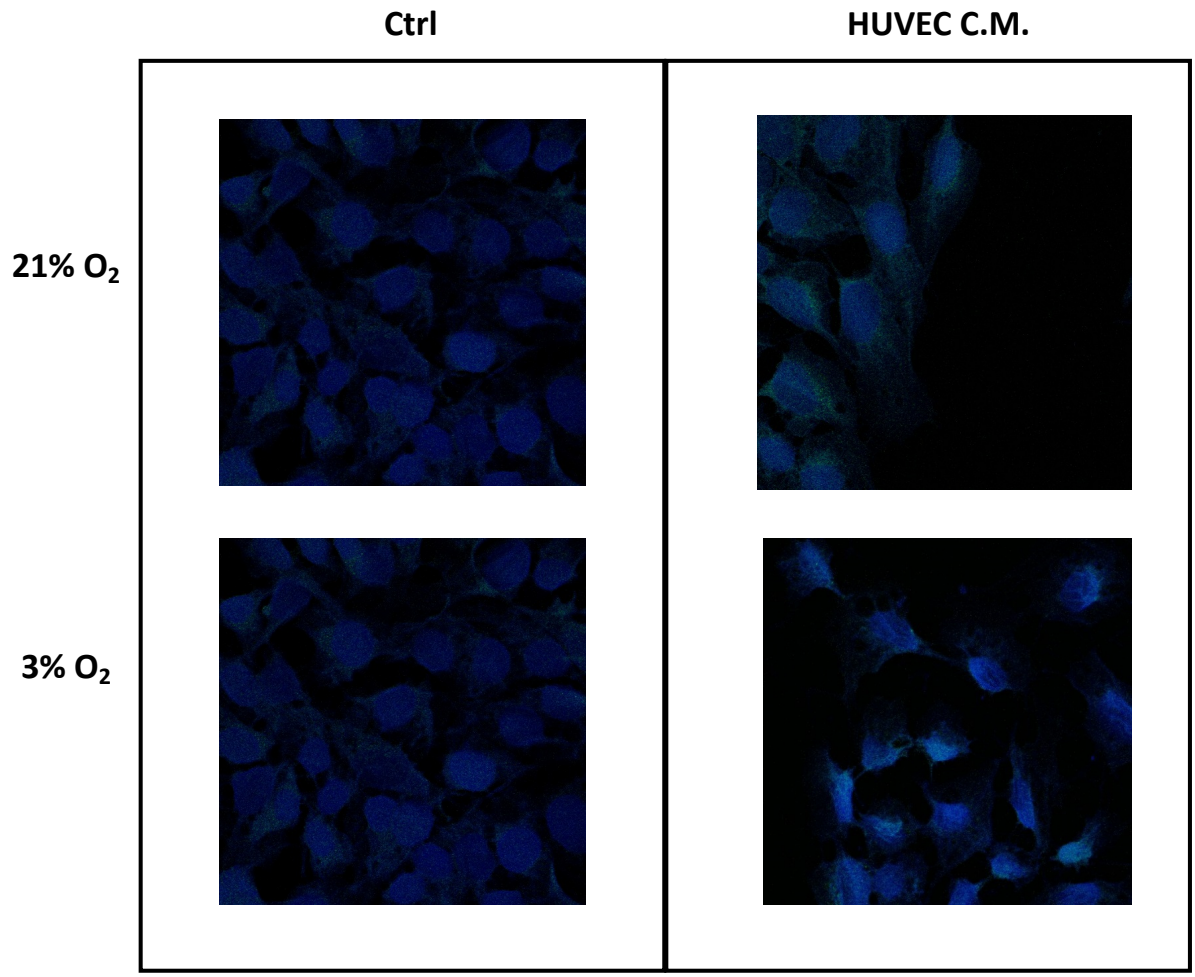


Figure 166

No CRHR2 was localized on adding HUVEC conditioned media on HTR8/SVneo cells under high and low oxygen tension at 24h. CRH : corticotrophin releasing hormone; HUVEC C.M.: conditioned media obtained from HUVEC cell culture; DAPI: blue nuclei; CRHR2 : green Alexa fluor 488.

CRHR2 localization on adding HUVEC conditioned media on HTR8/SVneo cells under high and low oxygen tension at 24h.

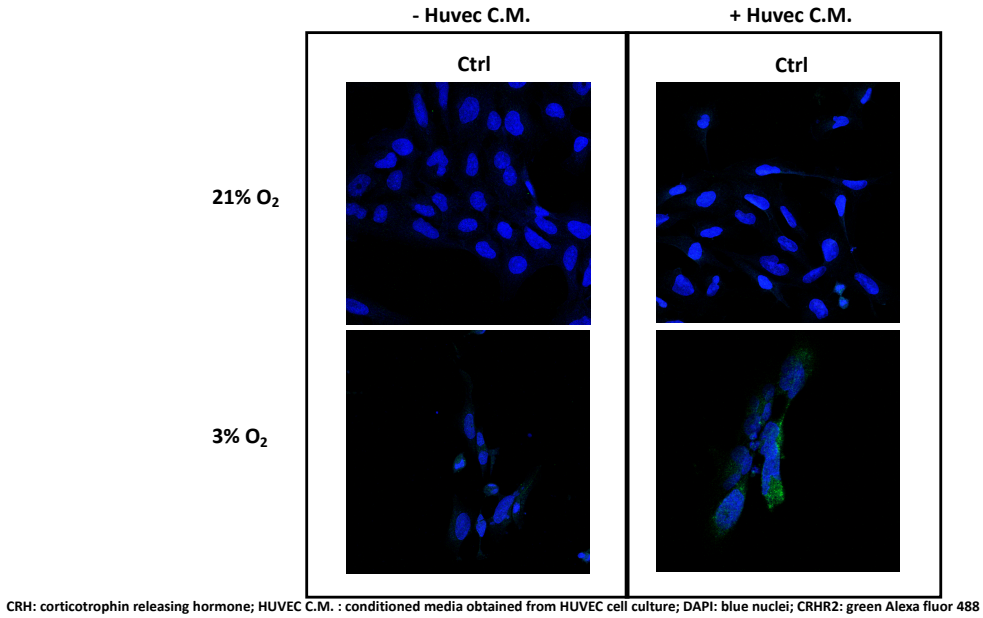


Figure 167

No CRHR2 was localized on adding HUVEC conditioned media on HTR8/SVneo cells under high and low oxygen tension at 24h. CRH : corticotrophin releasing hormone; HUVEC C.M.: conditioned media obtained from HUVEC cell culture; DAPI: blue nuclei; CRHR2 : green Alexa fluor 488.

CRHR2 localization on stimulating HTR8/SVneo cells with CRH under high and low oxygen tension at 15'.

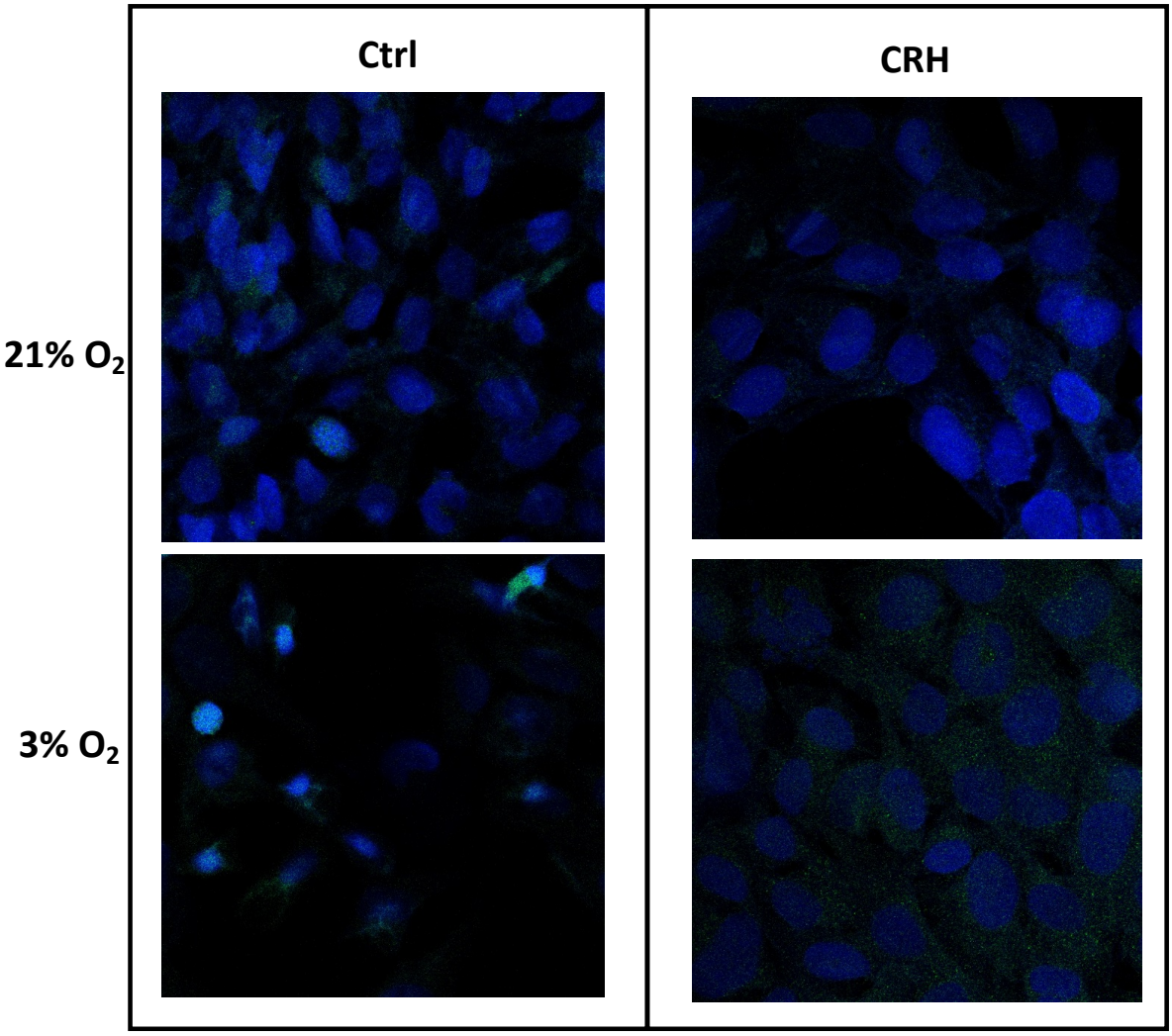


Figure 168

No CRHR2 was localized when HTR8/SVneo cells were treated with CRH under high and low oxygen tension at 15'. CRH : corticotrophin releasing hormone; HUVEC C.M.: conditioned media obtained from HUVEC cell culture; DAPI: blue nuclei; CRHR2 : green Alexa fluor 488.

CRHR2 localization on stimulating HTR8/SVneo cells with CRH under high and low oxygen tension at 24h.

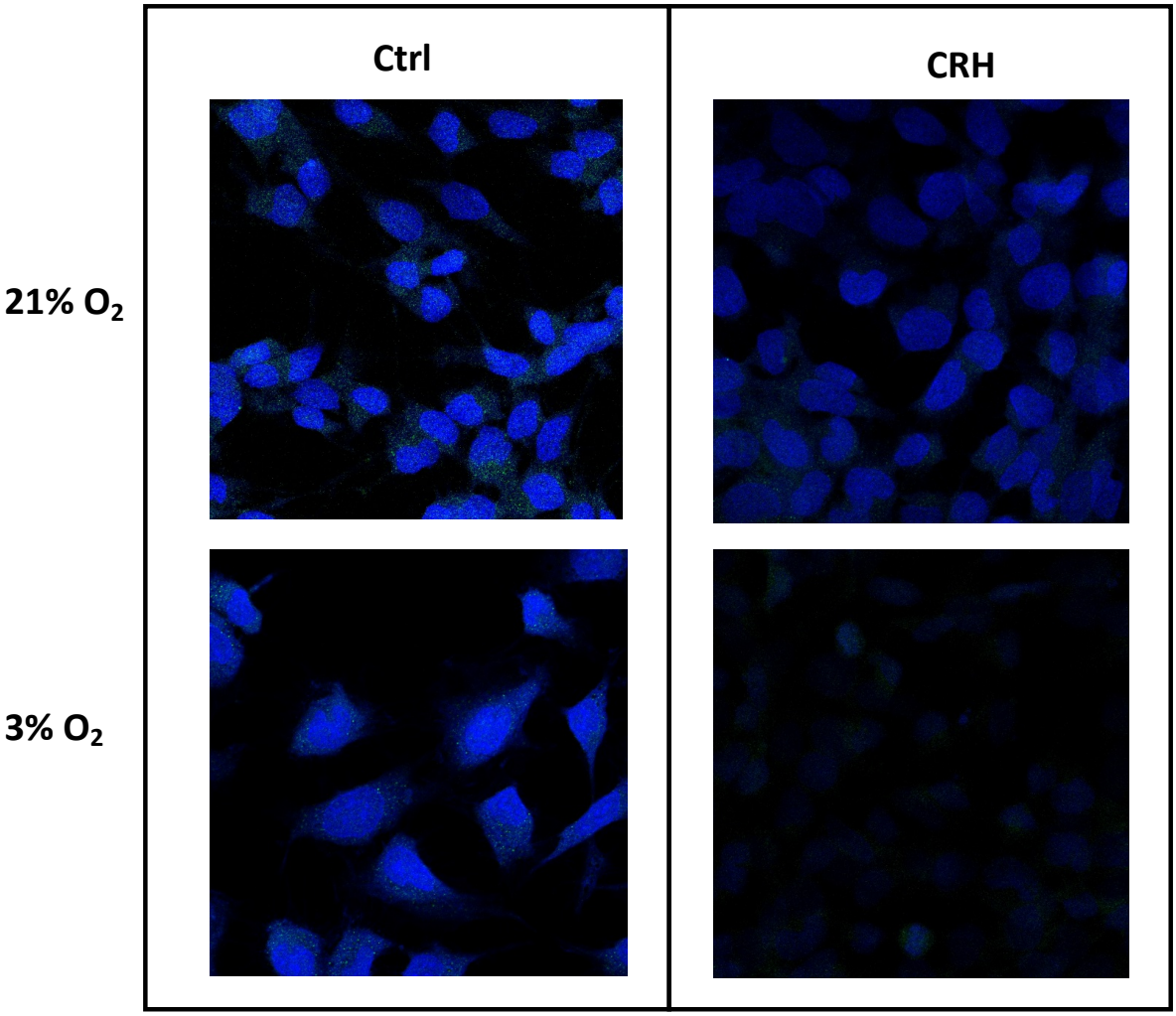


Figure 169

No CRHR2 was localized when HTR8/SVneo cells were treated with CRH under high and low oxygen tension at 24h. CRH : corticotrophin releasing hormone; HUVEC C.M.: conditioned media obtained from HUVEC cell culture; DAPI: blue nuclei; CRHR2 : green Alexa fluor 488.

CRHR2 localization on stimulating HTR8/SVneo cells with sFLT-1 under high and low oxygen tension at 24h.

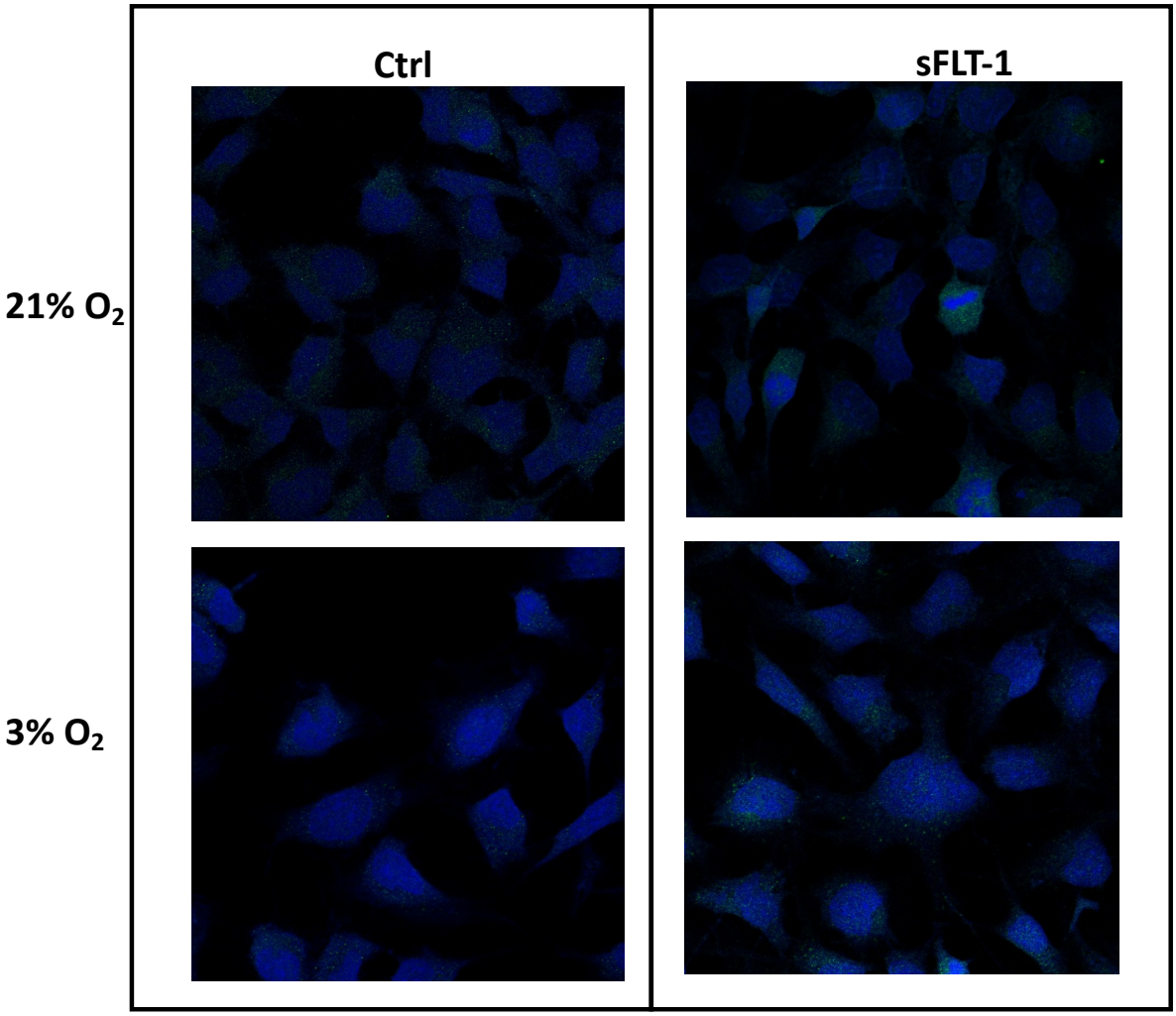


Figure 170

No CRHR2 was localized when HTR8/SVneo cells were treated with sFLT-1 under high and low oxygen tension at 24h. CRH : corticotrophin releasing hormone; HUVEC C.M.: conditioned media obtained from HUVEC cell culture; DAPI: blue nuclei; CRHR2 : green Alexa fluor 488.

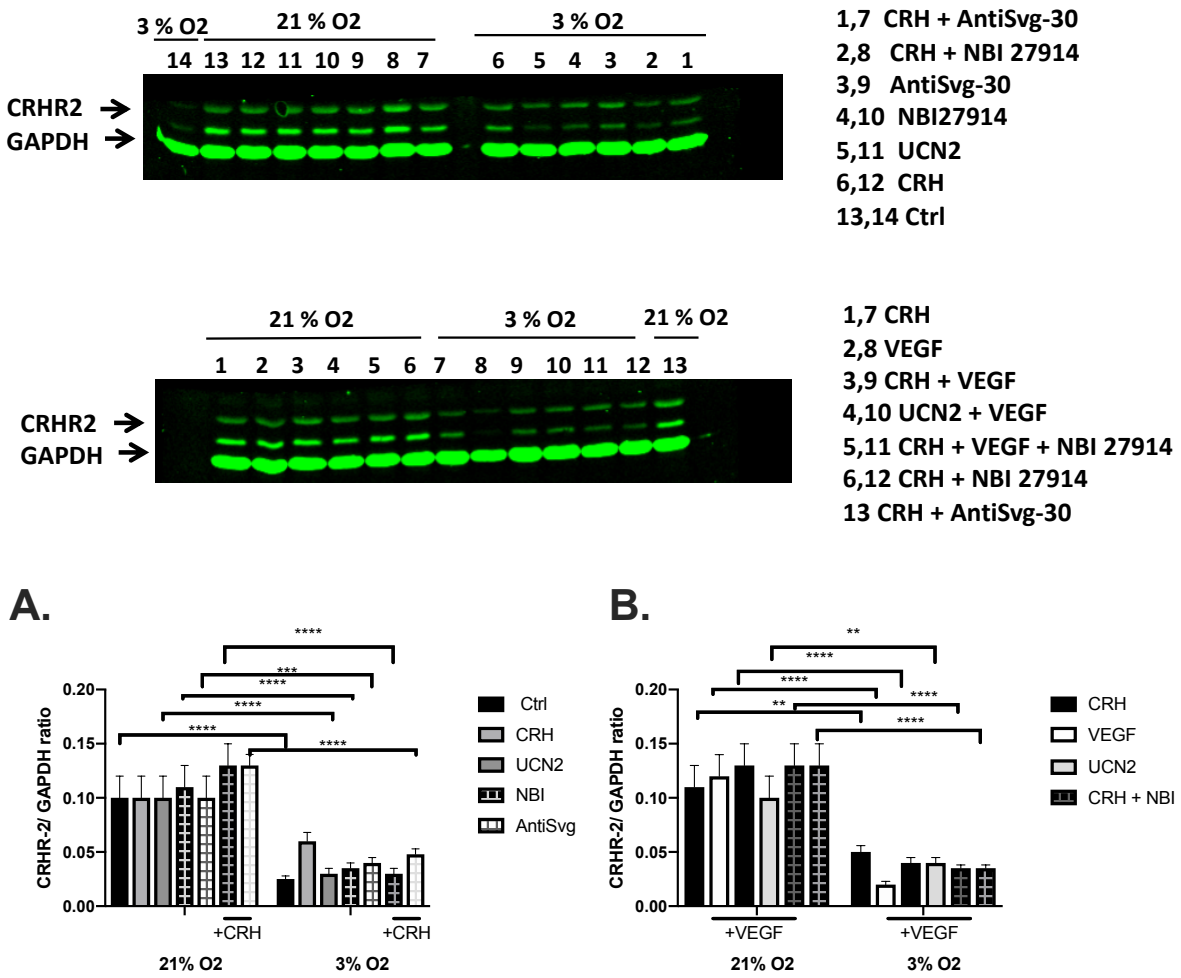


Figure 171

*Hypoxia reduces CRHR2 protein expression in HTR8/SVneo cells at 24h. Experiment protocol, where after 80% confluency, fresh media was added and HTR8/ SVneo cells were exposed to 3% O2 for 24h. Cells were pretreated with CRHR1 /2 antagonists, NBI 27914 or anti-sauvagine-30 for 1h. The cells were further treated with 100nM CRH for 24h and protein lysates were collected at 24h and western blot analysis was done on CRHR2 (40kDa) protein. Western blot analysis showed under low oxygen tension, CRHR2 protein expression is decreased in HTR8/SVneo cells at 24h. 25μg protein was loaded. All results are the mean (±SEM) /representative images of single experiment performed in triplicate(n=3). p=** .002 ;*** .0002 ;****< .0001; **** p<0.0001; n=3; CRH : corticotrophin releasing hormone; UCN2 : Urocortin 2; VEGF : Vascular endothelial growth factor; NBI : NBI-27914, Anti-Svg 30 : Anti-Sauvagine 30. Data expressed as (A) fold change of protein expression, relative to control 24h, after normalising total protein against GAPDH. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

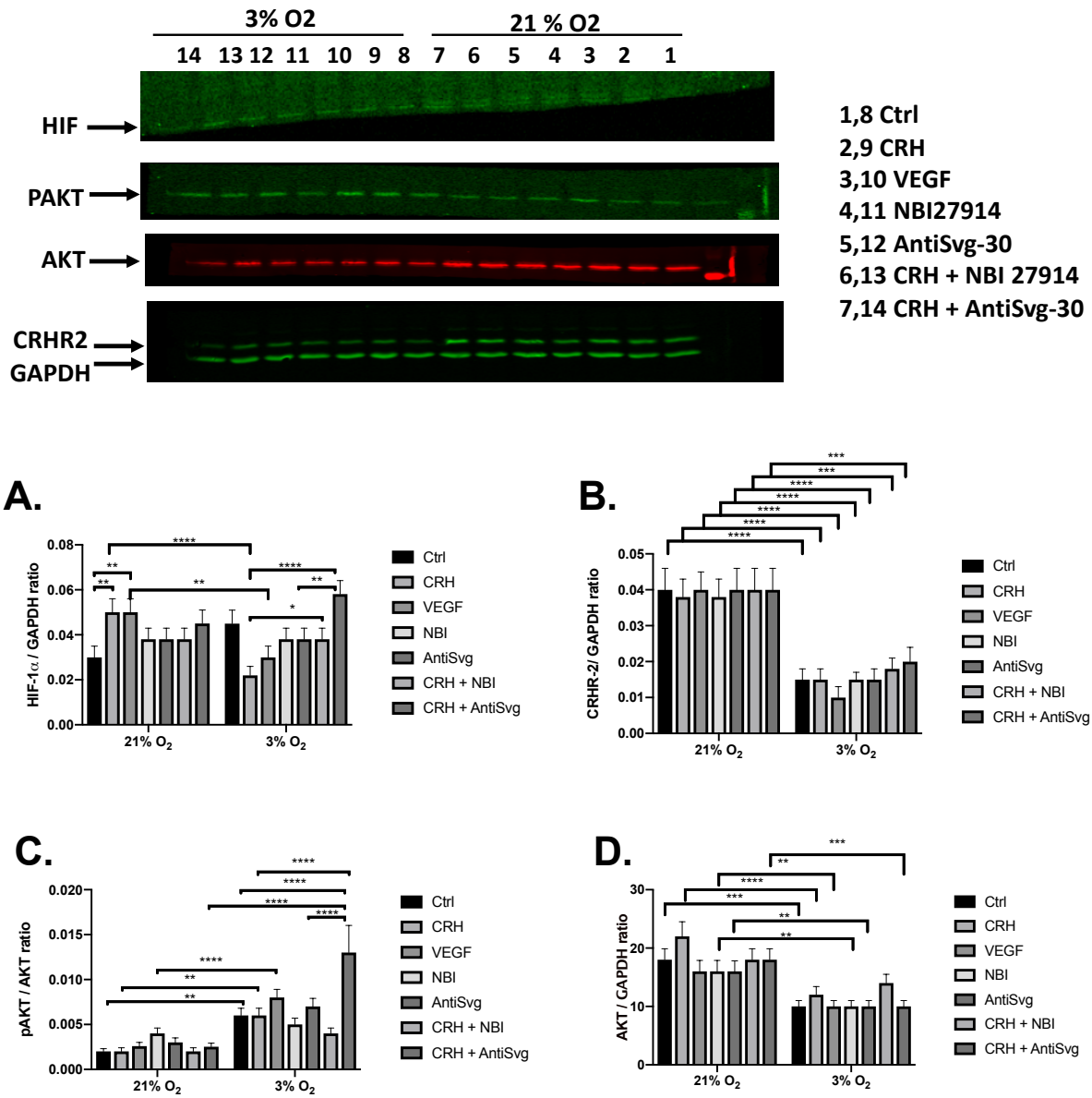
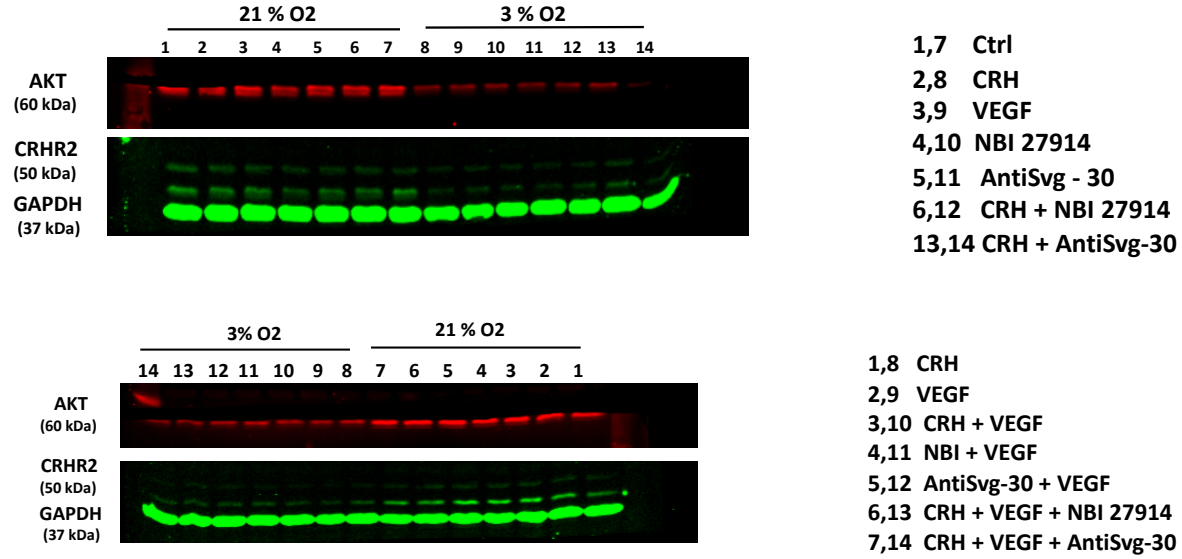


Figure 172

*Hypoxia increases pAKT and decreases AKT, CRHR2 protein expression in HTR8/SVneo cells at 24h. Under low oxygen tension, CRH reduces HIF-1a protein expression via CRHR2. Experiment protocol, where after 80% confluency, fresh media was added and HTR8/ SVneo cells were exposed to 3% O2 for 24h. Cells were pretreated with CRHR1 /2 antagonists, NBI 27914 or anti-sauvagine-30 for 1h. The cells were further treated with 100nM CRH for 24h and protein lysates were collected at 24h and western blot analysis was done on HIF-1a (120kDa), CRHR2 (40kDa), pAKT (60kDa) and AKT (60kDa) protein. Western blot analysis showed under (A) Under high oxygen tension, CRH increases HIF-1a. VEGF increases HIF-1a. Low oxygen tension increases HIF-1a. Under low oxygen tension, CRH reduces HIF-1a via CRHR2. Blocking CRHR1 under high or low oxygen tension causes no change in CRH mediated regulation of HIF-1a. Blocking CRHR2 under low oxygen tension reverses the effect of hypoxia and causes CRH to increase HIF-1a expression. (B) Hypoxia decreases CRHR2 expression in HTR8 cells. (C) Hypoxia increases pAKT expression. Blocking CRHR2 causes CRH to increase pAKT expression. (D) Hypoxia decreases AKT expression. 25μg protein was loaded. All results are the mean (±SEM) /representative images of single experiment performed in triplicate(n=3). p=** .002 ;*** .0002 ;****< .0001; **** p<0.0001; n=3; CRH : corticotrophin releasing hormone; VEGF : Vascular endothelial growth factor; NBI : NBI-27914, Anti-Svg 30 : Anti-Sauvagine 30. Data expressed as (A) fold change of protein expression, relative to control 24h, after normalising total protein against GAPDH. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

Htr8 24h



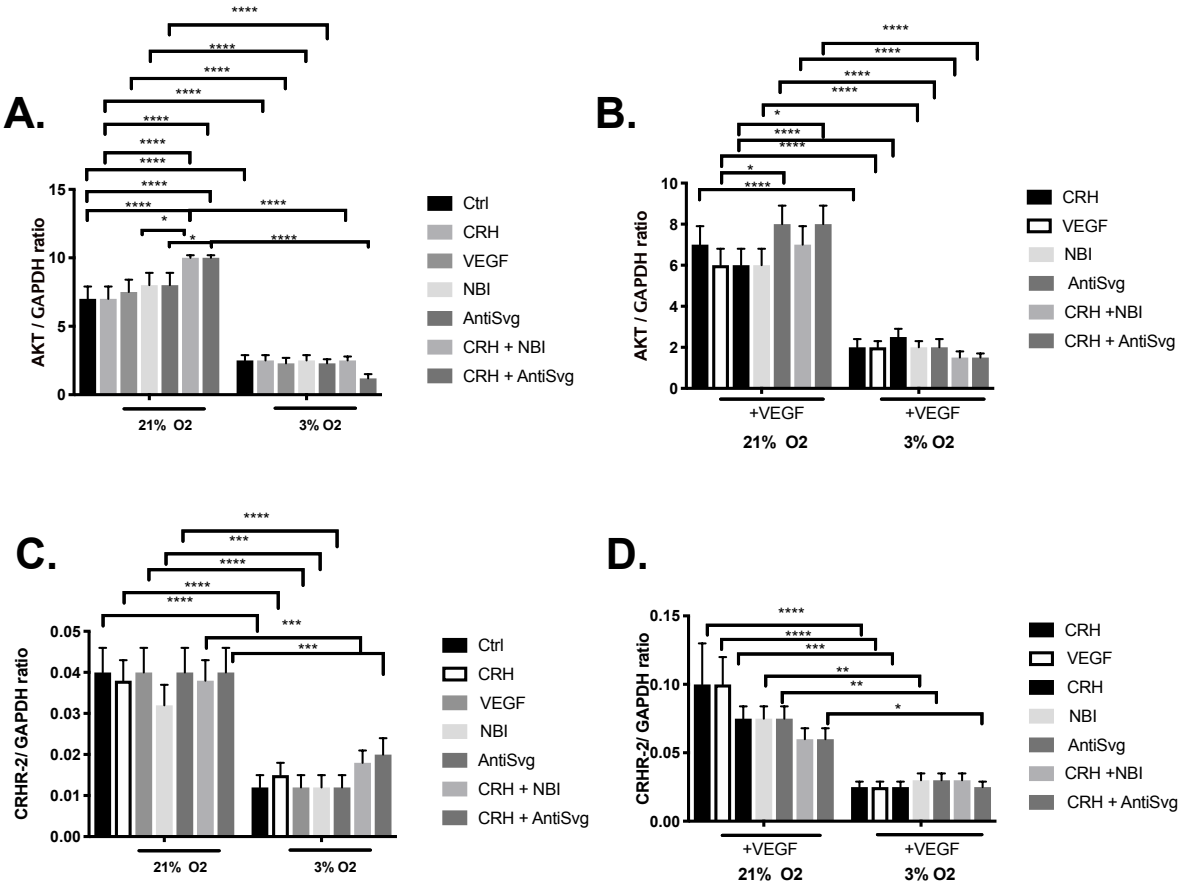


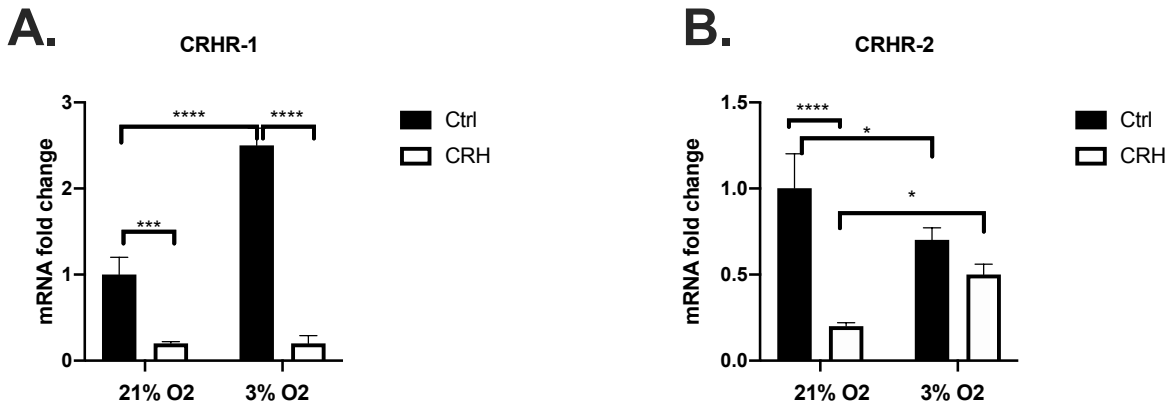
Figure 173

Hypoxia decreases protein expression of AKT and CRHR2 in HTR8/SVneo cells. Experiment protocol, where after 80% confluency, fresh media was added and HTR8/ SVneo cells were exposed to 3% O2 for 24h. Cells were pretreated with CRHR1 /2 antagonists, NBI 27914 or anti-sauvagine-30 for 1h. The cells were further treated with 100nM CRH for 24h and protein

*lysates were collected at 24h and western blot analysis was done on CRHR2 (40kDa), and AKT (60kDa) protein. Western blot analysis showed under under low oxygen tension, the protein expression of (A) AKT and (C) CRHR2 are reduced in HTR8/SVneo cells at 24h. 25μg protein was loaded. All results are the mean (±SEM) /representative images of single experiment performed in triplicate(n=3). p=** .002 ;*** .0002 ;****< .0001; **** p<0.0001; n=3; CRH : corticotrophin releasing hormone; VEGF : Vascular endothelial growth factor; NBI : NBI-27914, Anti-Svg 30 : Anti-Sauvagine 30. Data expressed as (A) fold change of protein expression, relative to control 24h, after normalising total protein against GAPDH. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

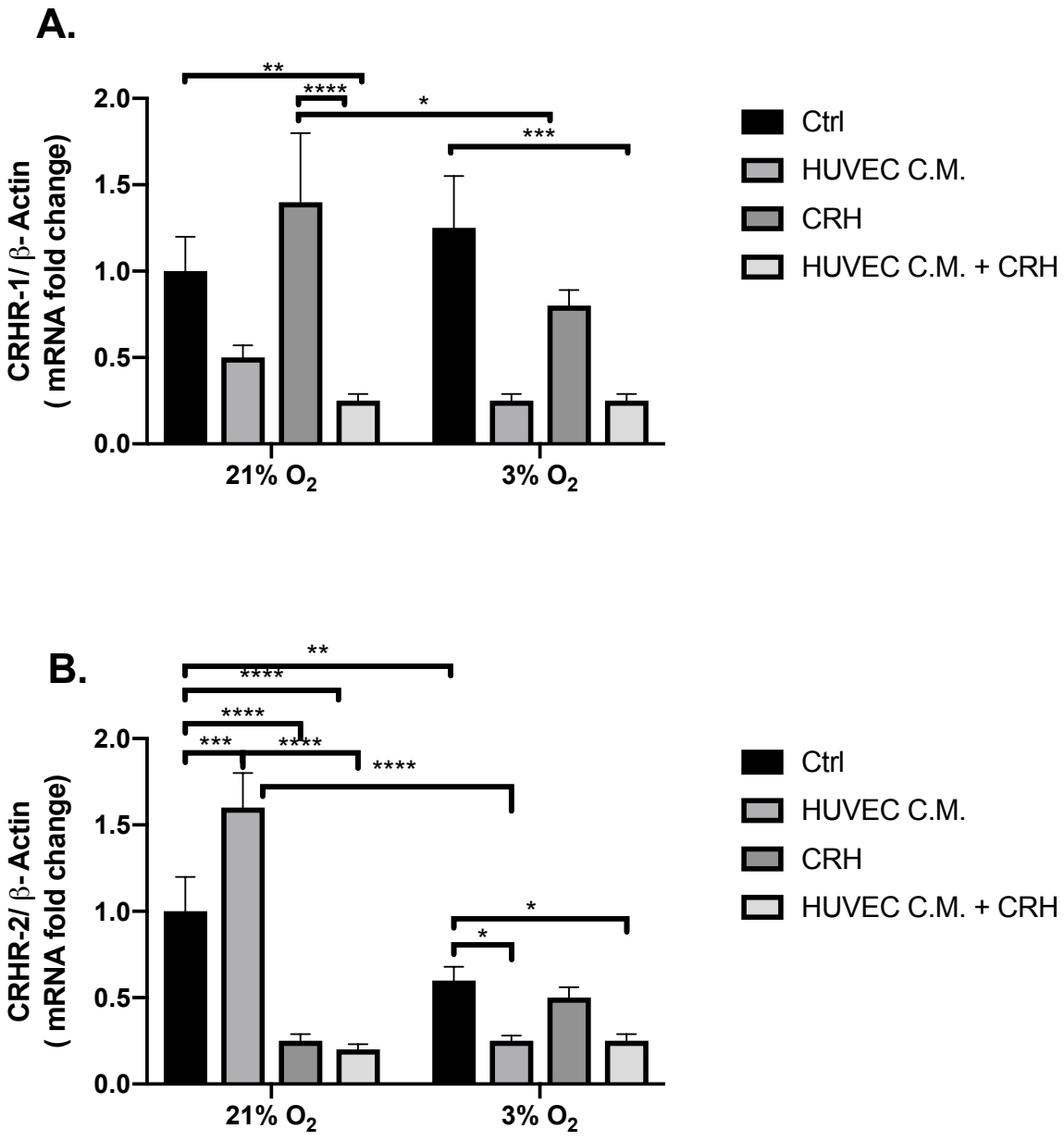
HTR8 24H

The regulation of CRHRs expression by CRH under high and low oxygen tension in HTR8 / SV neo cells at 24h.



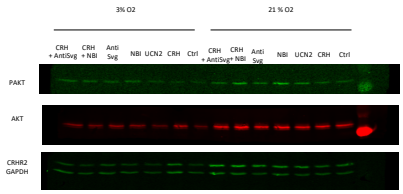
Under high oxygen tension, CRH reduces CRHR1 and CRHR2 mRNA expression in HTR8/SV neo cells at 24h. Hypoxia upregulates CRHR1 expression and downregulates CRHR2 mRNA expression. CRH downregulates CRHR2 mRNA expression under low oxygen tension.

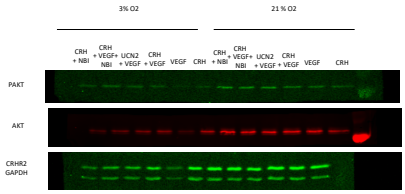
The effect of CRH on co-culture between HTR8/SVneo cells and HUVECs under low oxygen tension



Hypoxia reduces CRHR2 mRNA expression. HUVEC C.M. increases CRHR2 mRNA expression in HTR8/SV neo cells at 24h under high oxygen tension, which is reversed under low oxygen tension.

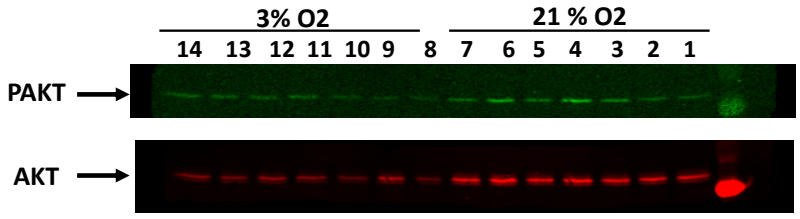
Htr8 1h



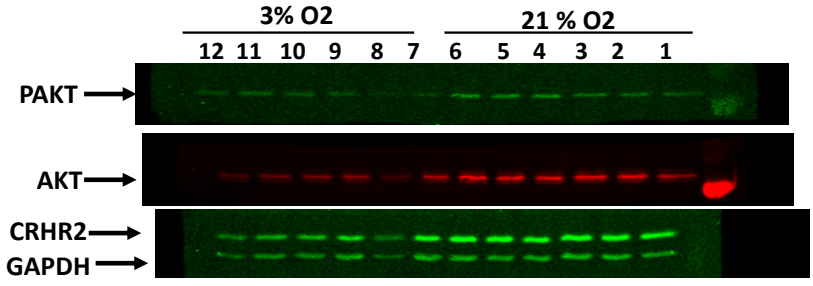


05/02/2019

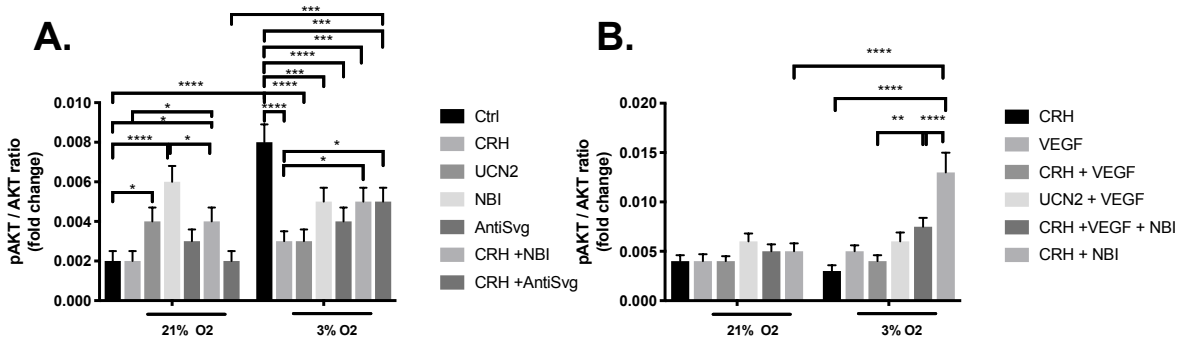
HTR8, 1H 45



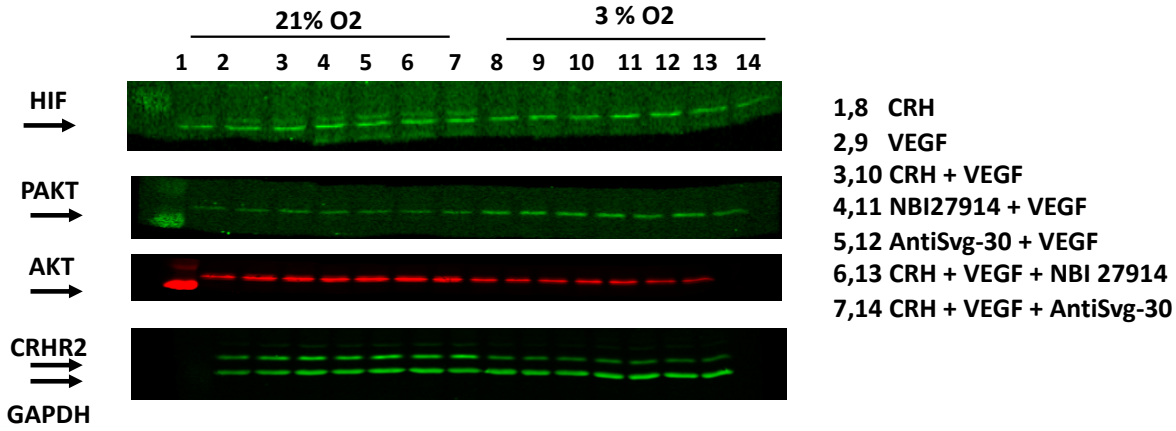
1,8 Ctrl
2,9 CRH
3,10 UCN2
4,11 NBI27914
5,12 AntiSvg-30
6,13 CRH + NBI 27914
7,14 CRH + AntiSvg-30

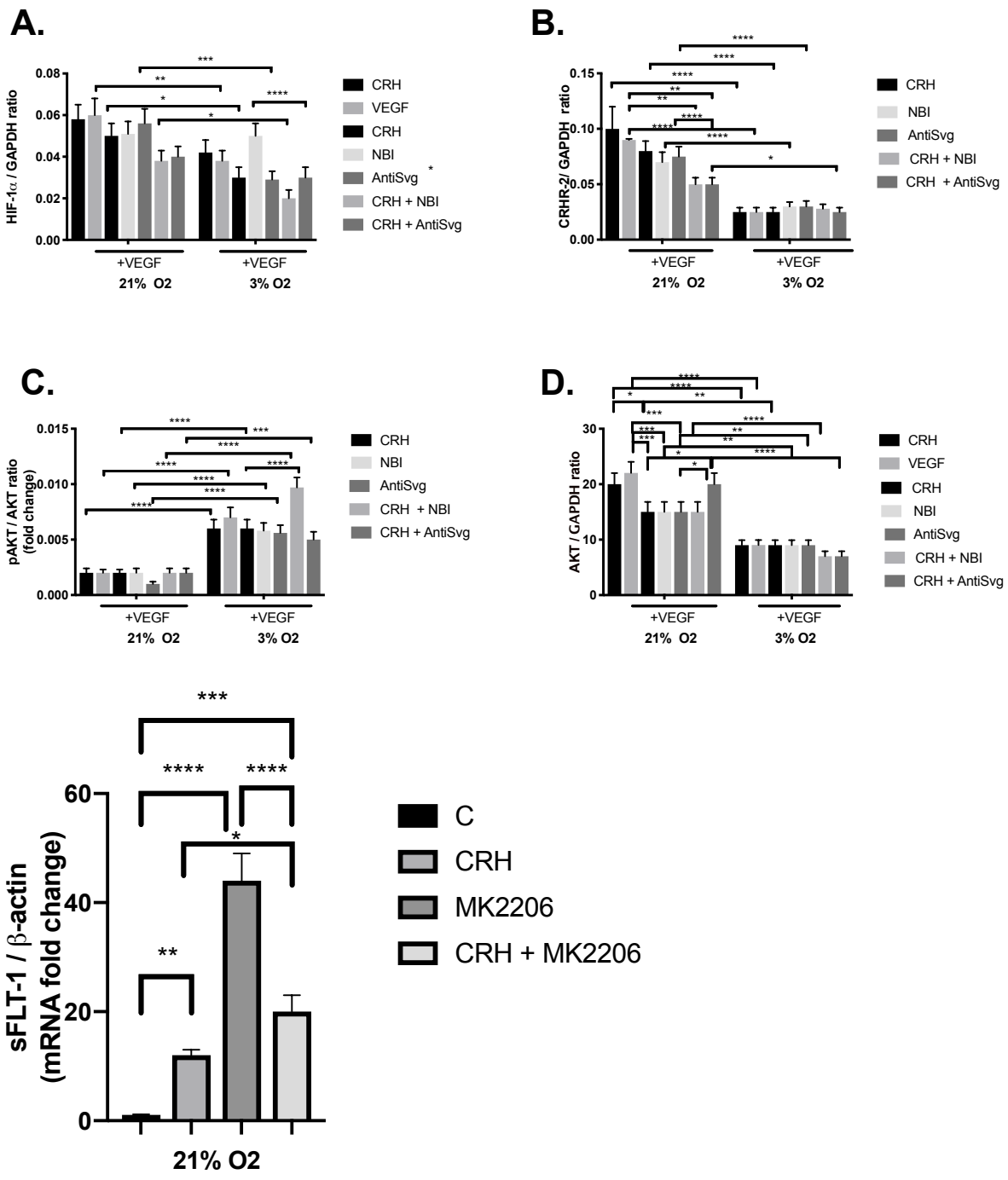


1,7 CRH
2,8 VEGF
3,9 CRH + VEGF
4,10 UCN2 + VEGF
5,11 CRH + VEGF + NBI 27914
6,12 CRH + NBI 27914


A.
pAKT / AKT ratio (fold change)
0.010
0.008
0.006
0.004
0.002
0.000
21% O2
3% O2
Ctrl
CRH
UCN2
NBI
AntiSvg
CRH +NBI
CRH +AntiSvg
B.
pAKT / AKT ratio (fold change)
0.020
0.015
0.010
0.005
0.000
21% O2
3% O2
CRH
VEGF
CRH + VEGF
UCN2 + VEGF
CRH +VEGF + NBI
CRH + NBI

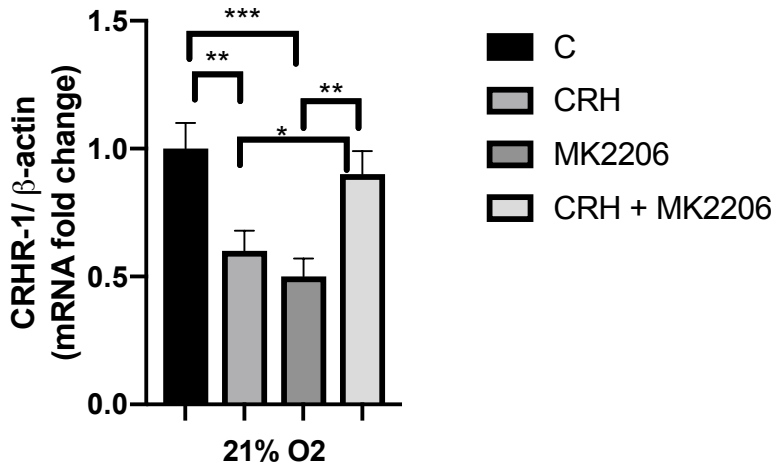
HTR8 24H


21% O2
3 % O2
1 2 3 4 5 6 7 8 9 10 11 12 13 14
HIF
PAKT
AKT
CRHR2
GAPDH
1,8 CRH
2,9 VEGF
3,10 CRH + VEGF
4,11 NBI27914 + VEGF
5,12 AntiSvg-30 + VEGF
6,13 CRH + VEGF + NBI 27914
7,14 CRH + VEGF + AntiSvg-30



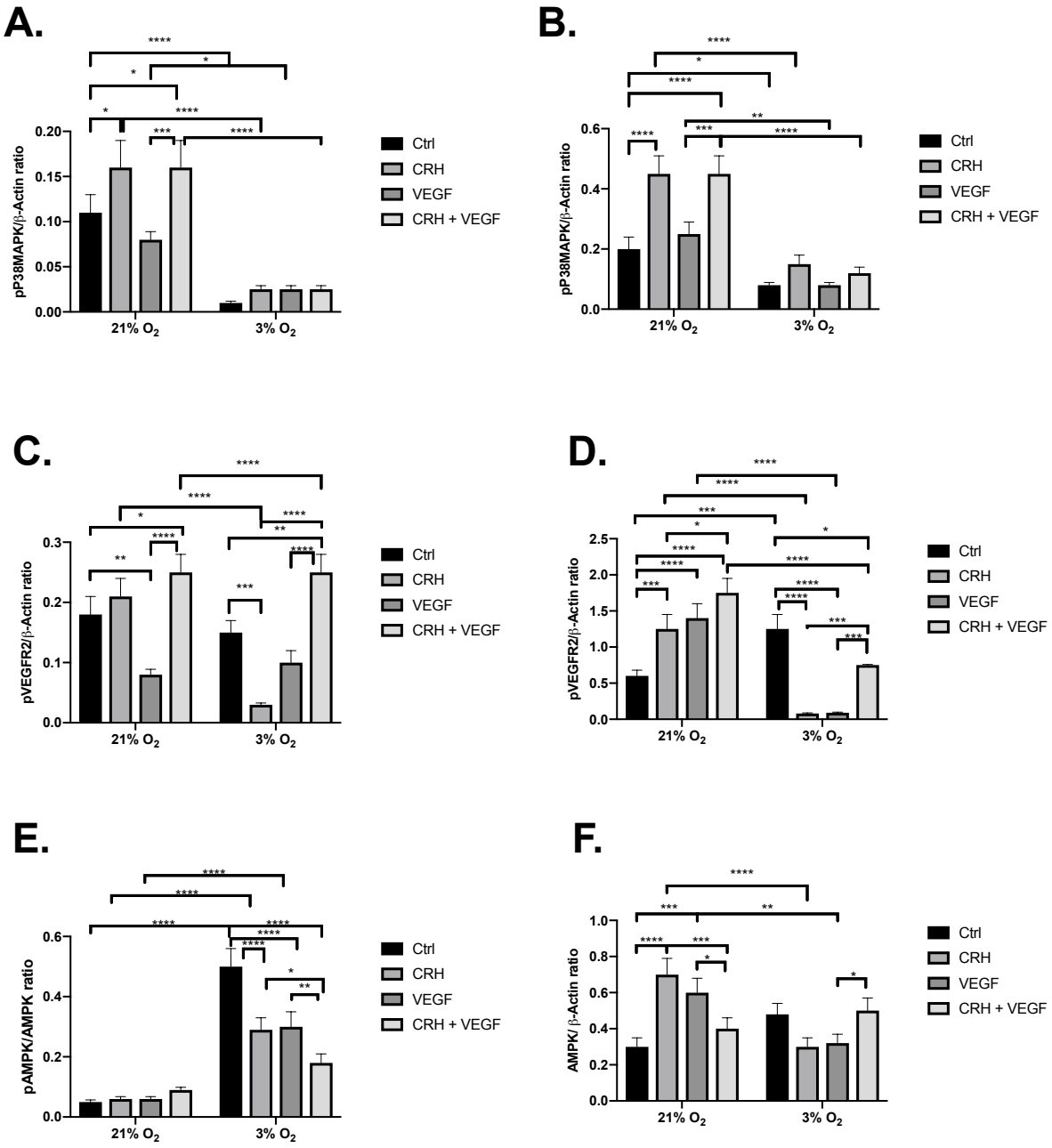
100nM CRH, MK2206 5uM

CRH upregulates sFLT-1 mRNA expression in HTR8/SV neo cells under high oxygen tension at 24h. CRH downregulates sFLT-1 mRNA expression when combined with AKT inhibitor (MK 2206).

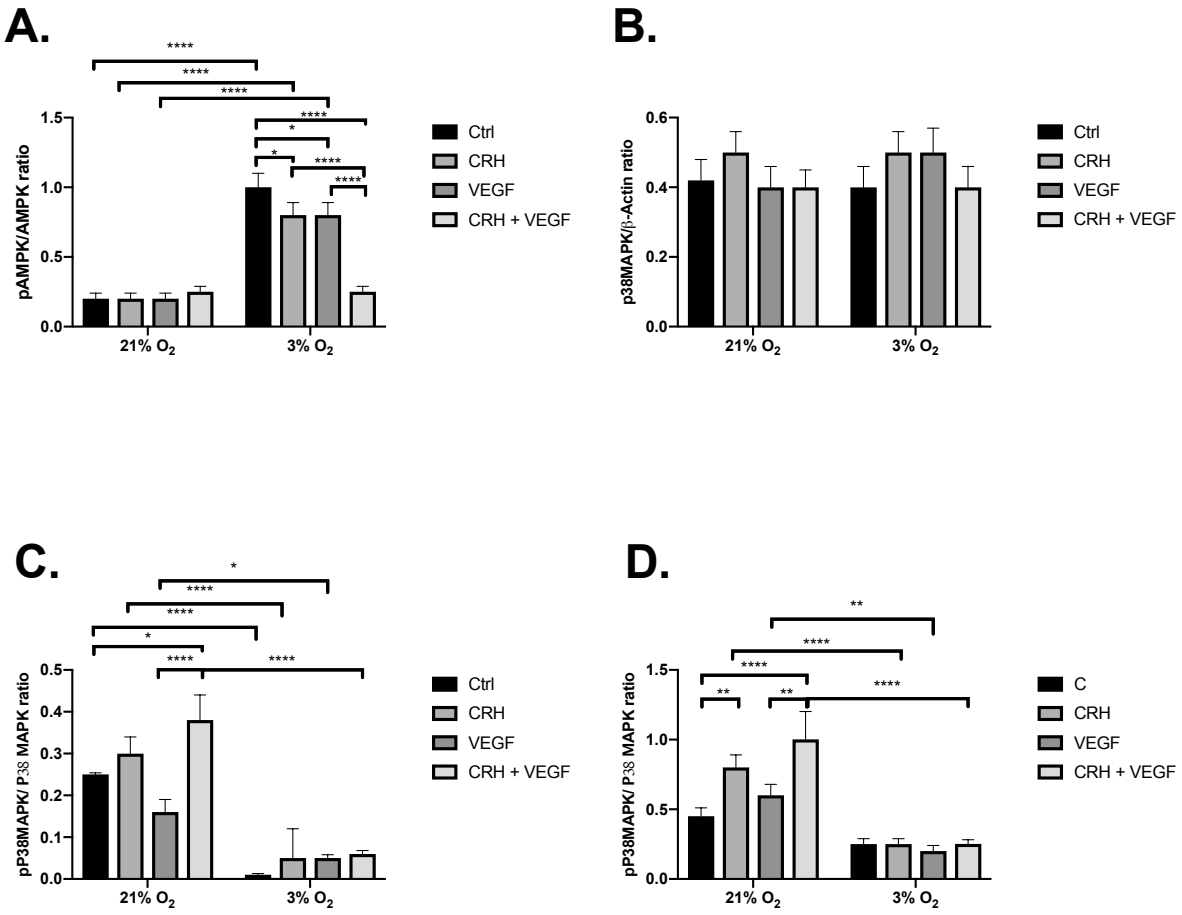


CRH downregulates CRHR1 mRNA expression in HTR8/SV neo cells at 24h. CRH upregulates CRHR-1 mRNA expression when combined with AKT inhibitor (MK 2206).

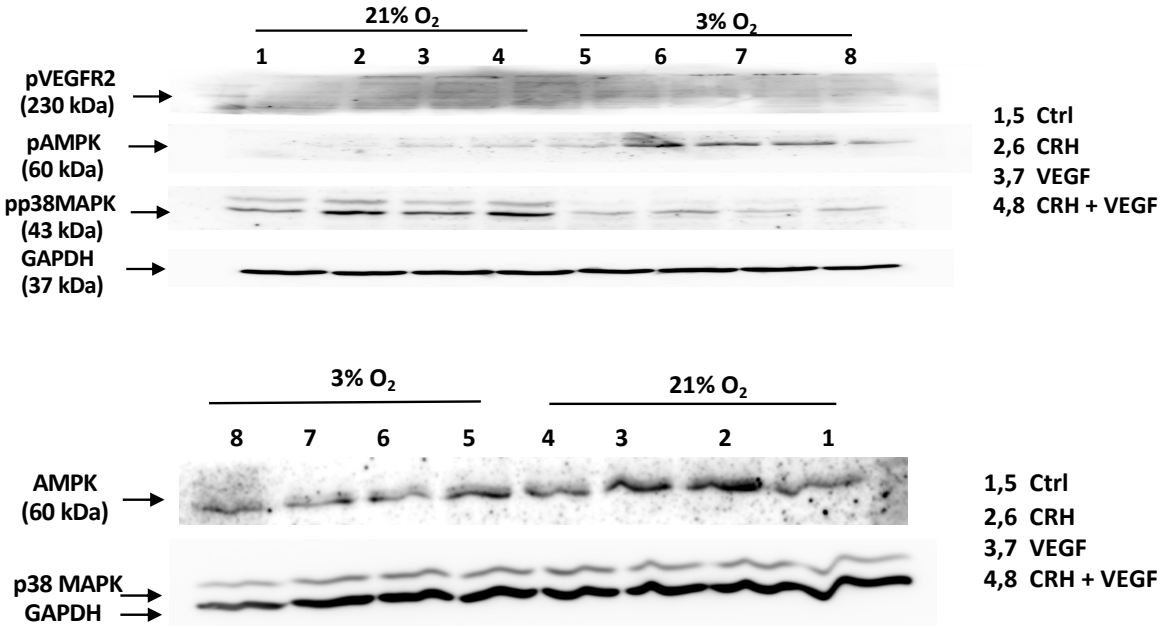
HTR8 15’


A.
B.
C.
D.
E.
F.
pP38MAPK/β-Actin ratio
pVEGFR2/β-Actin ratio
pAMPK/AMPK ratio
AMPK/ β-Actin ratio
21% O2
3% O2
Ctrl
CRH
VEGF
CRH + VEGF

HTR8 15’

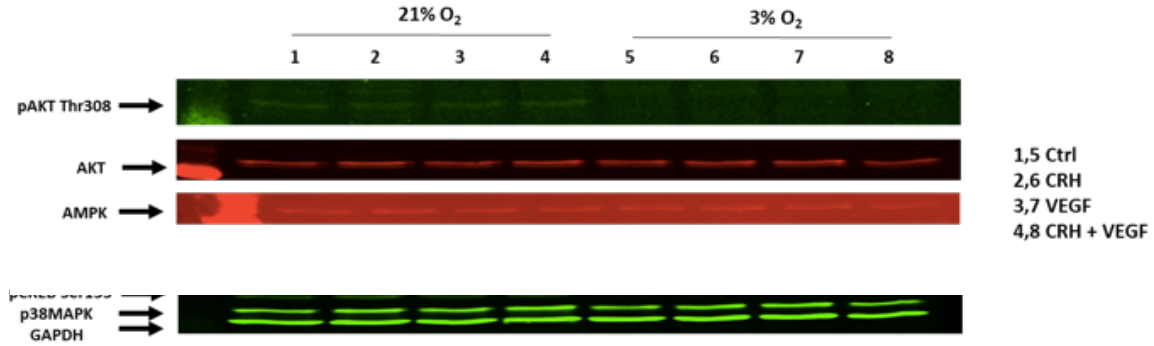

A.
pAMPK/AMPK ratio
21% O2
3% O2
Ctrl
CRH
VEGF
CRH + VEGF
B.
p38MAPK/β-Actin ratio
C.
pP38MAPK/ P38 MAPK ratio
D.
C

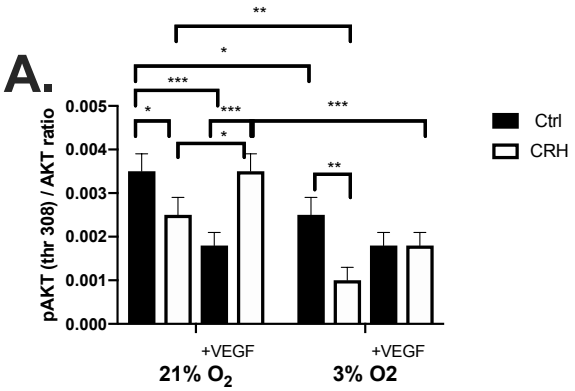
Htr8 15’

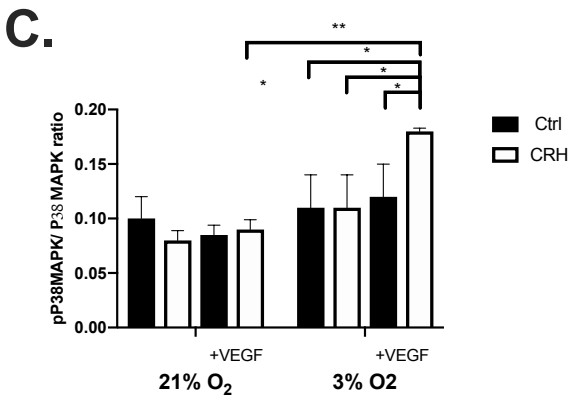

21% O2
3% O2
1 2 3 4 5 6 7 8
pVEGFR2 (230 kDa)
pAMPK (60 kDa)
pp38MAPK (43 kDa)
GAPDH (37 kDa)
1,5 Ctrl
2,6 CRH
3,7 VEGF
4,8 CRH + VEGF
3% O2
21% O2
8 7 6 5 4 3 2 1
AMPK (60 kDa)
p38 MAPK
GAPDH
1,5 Ctrl
2,6 CRH
3,7 VEGF
4,8 CRH + VEGF

Western blot (ECL) analysis of effect of CRH on phosphorylation of VEGFR2 (55B11) , AMPK (Thr172), p38 MAPK (Thr 180 / Tyr 182) under high and low oxygen tension in HTR8 / SVneo cells at 15'.

Htr815'

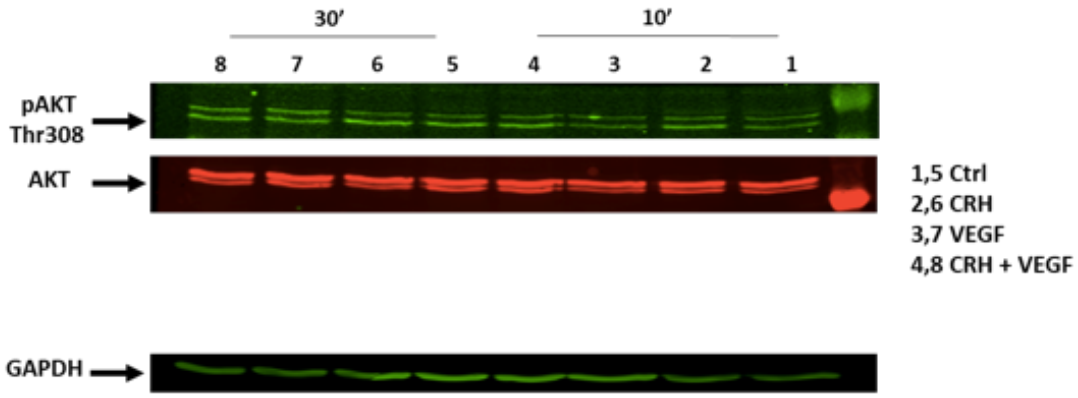


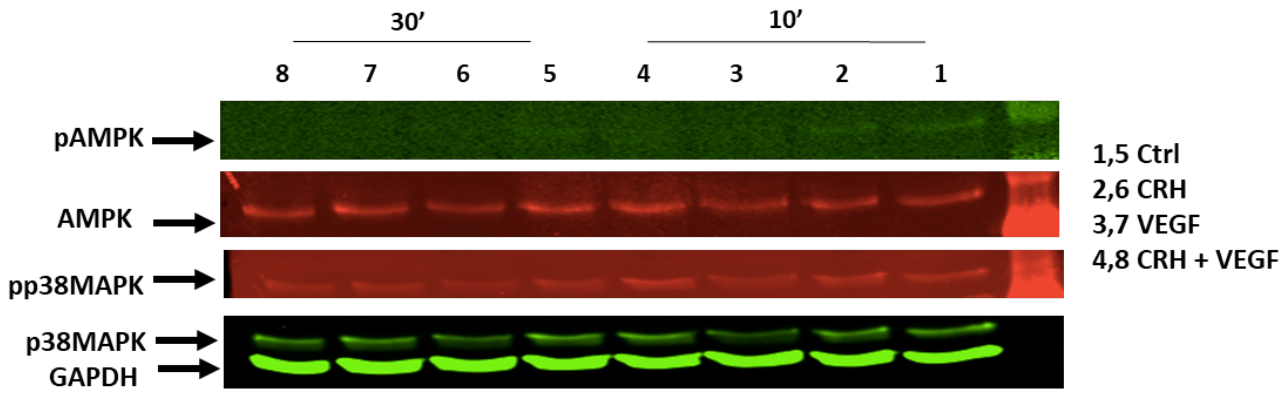


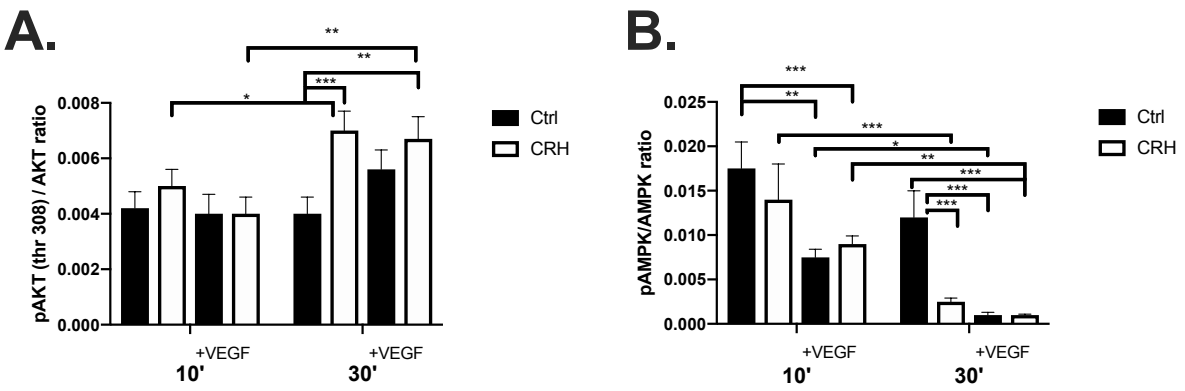


Western blot (Licor) analysis of effect of CRH on phosphorylation of VEGFR2 (55B11) , AMPK (Thr172), p38 MAPK (Thr 180 / Tyr 182) under high and low oxygen tension in HTR8 / SVneo cells at 15'. Under high oxygen tension, CRH and VEGF reduces pAKT (thr 308) in HTR8/SV neo cells at 15'. CRH reverses VEGF mediated regulation of pAKT (thr 308). Hypoxia reduces pAKT (thr 308). Under low oxygen tension, CRH reduces pAKT (thr 308). Under low oxygen tension, CRH potentiates VEGF mediated regulation of pP38 MAPK.

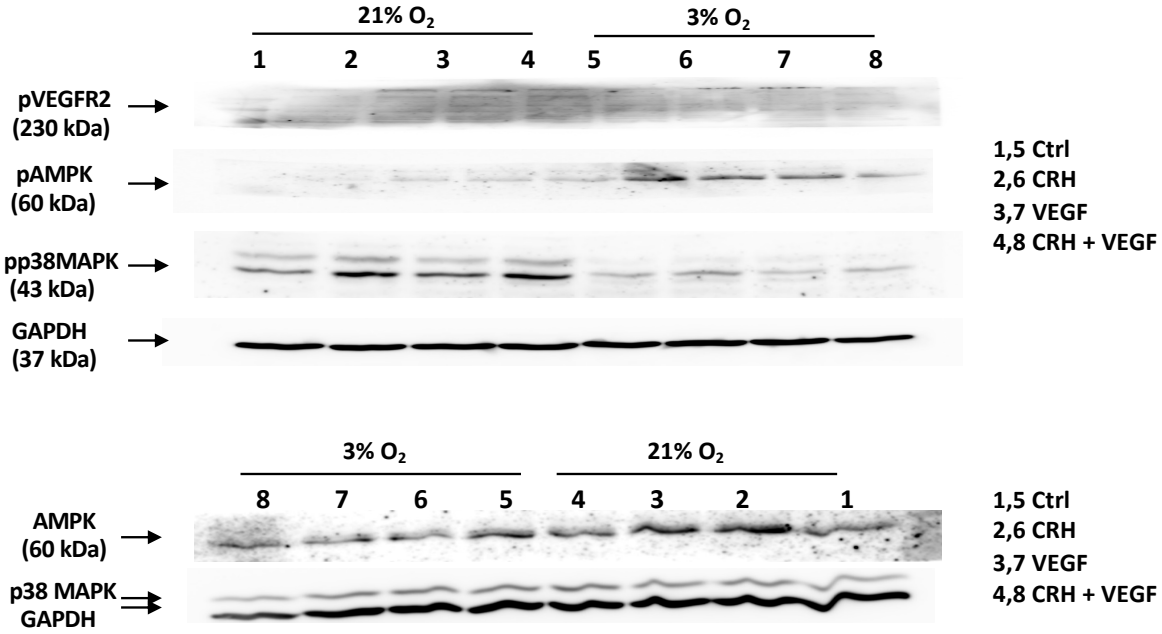
The effect of CRH on phosphorylation of AKT , AMPK, p38 MAPK in HTR8/SV neo cells under high and low oxygen tension

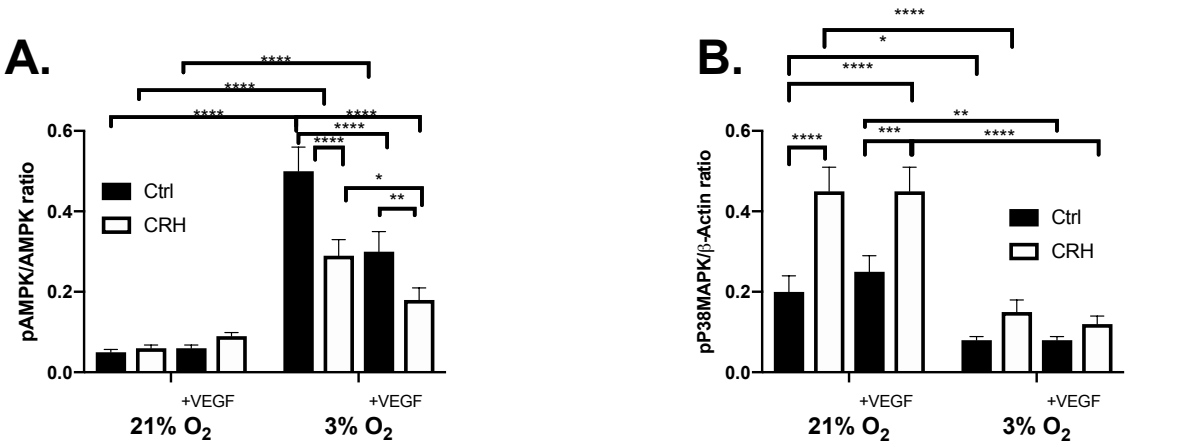






Under high oxygen tension, CRH increases pAKT (thr 308) at 30'. VEGF reduces pAMPK (Thr 172) in a time dependent manner. Under low oxygen tension, CRH reduces p-AMPK at 30'. pP38 MAPK data was non significant (not shown).





*Under high oxygen tension, CRH increases p-p38 MAPK in HTR8/SVneo cells at 1h. Under low oxygen tension, CRH reduces pAMPK protein expression and CRH loses its ability to increase p-p38 MAPK.Experiment protocol, where after 80% confluency, fresh media was added and HTR8/ SVneo cells were exposed to 3% O2 for 24h. Cells were pretreated with CRHR1 /2 antagonists, NBI 27914 or anti-sauvagine-30 for 1h. The cells were further treated with 100nM CRH for 24h and protein lysates were collected at 24h and western blot analysis was done on pVEGFR2 (230kDa), CRHR2 (40kDa), AMPK (60kDa), p38 MAPK (43kDa) protein. Western blot analysis showed hypoxia increases phosphorylation of AMPK in HTR8/SVneo cells. under low oxygen tension, VEGF reduces pAMPK. CRH reduces pAMPK and VEGF mediated regulation of pAMPK under low oxygen tension. CRH increases phosphorylation of P38 MAPK in HTR8/SVneo cells under high oxygen tension. Hypoxia reduces p-p38 MAPK in HTR8/SVneo cells and CRH loses its ability to increase p-p38 MAPK under low oxygen tension. VEGF has no effect on p-p38 MAPK under high and low oxygen tension. 25µg protein was loaded. All results are the mean (±SEM) /representative images of single experiment performed in triplicate(n=3). p=** .002 ;*** .0002 ;****< .0001; **** p<0.0001; n=3; CRH : corticotrophin releasing hormone; VEGF : Vascular endothelial growth factor; NBI : NBI-27914, Anti-Svg 30 : Anti-Sauvagine 30. Data expressed as (A) fold change of protein expression, relative to control 24h, after normalising total protein against GAPDH. Data were analysed using a two-way ANOVA with Tukey's multiple comparison test.*

6.3 HUVEC

Table 64

sFLT-1 mRNA	21% O2				3% O2			
	Ctrl	CRH	UCN2	VEGF	Ctrl	CRH	UCN2	VEGF
HUVEC	NS	↓	NS	NS	NS	NS	NS	NS
HMEC-1	NS	NS	NS	↑	NS	NS	NS	NS
HTR8 /SVneo	NS	NS	NS	NS	NS	NS	NS	NS
BEWO	NS	NS	NS	NS	NS	NS	NS	NS

CRH reduces sFLT-1 mRNA expression in HUVECs and VEGF upregulates sFLT-1 mRNA expression in HMEC-1. Hypoxia abolishes these effects. UCN2 has no effect on sFLT-1 mRNA under high and low oxygen tension.

Table 65

sFLT-1 release	21% O2				3% O2			
	Ctrl	CRH	UCN2	VEGF	Ctrl	CRH	UCN2	VEGF
HUVEC	NS	↓	↓	↓	NS	NS	NS	NS
HMEC-1	NS	NS	↑	NS	NS	NS	NS	NS
HTR8 /SVneo	NS	NS	NS	NS	↑	↑	NS	NS

CRH, UCN2 and VEGF reduces sFLT-1 release in HUVECs. UCN2 increases sFLT-1 release in HMEC-1. Hypoxia abolishes these effects. Hypoxia increases sFLT-1 release in HTR8 / SVneo cells. CRH increases sFLT-1 release in HTR8/SVneo cells.

Table 66

PLGF release	21% O2				3% O2			
	Ctrl	CRH	UCN2	VEGF	Ctrl	CRH	UCN2	VEGF
HUVEC	NS	↓	↓	↑	NS	NS	NS	NS
HMEC-1	NS	NS	↑	↑	NS	NS	NS	NS
HTR8 /SVneo	NS	NS	NS	NS	NS	↑	NS	NS

CRH and UCN2 reduces PLGF secretion in HUVEC. VEGF increases PLGF secretion in HUVEC. Hypoxia abolishes the effect. UCN2 and VEGF increases PLGF secretion in HMEC-1. CRH increases PLGF secretion in HTR8/SVneo cells under low oxygen tension.

The regulation of sFLT-1 mRNA expression by CRH in HUVEC cells under low oxygen tension

Figure 174

*Hypoxia abolishes the CRH induced reduction in sFLT-1 mRNA expression in HUVEC cells. QRT PCR analysis showed that the mRNA expression of sFLT-1 by CRH was (A) significantly downregulated at 21% O2; and (B)unaffected at 3% O2 for 24h. *p= 0.033; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using a two-tailed unpaired t-test.*

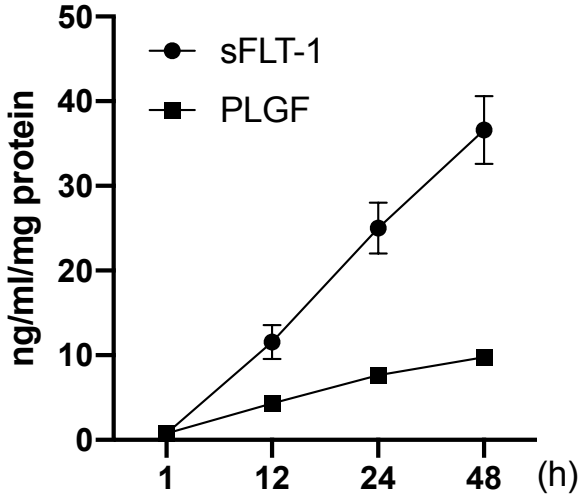


Figure 175

*Under high oxygen tension, sFLT-1 and PLGF are endogenously secreted in a time dependent manner upto 48h in HUVEC cells, the former being released in excess. Experiment protocol, where after 80% confluency, fresh media was added and supernatants were collected at different time points and MSD ELISA assay was done on sFLT-1 and PLGF proteins.Both sFLT-1 and PLGF proteins were significantly released in a time dependent manner upto 48 h from HUVEC cells at 21% O2, the former being released in higher amounts. SFLT-1 protein was released in minimal amounts endogenously. p=0.1234 ns ; * 0.033; ** .002; *** .0002; ****< .0001; n=3.Data expressed as ng/ml normalised against mg protein. Data were analysed using one – way ANOVA with Tukeys multiple comparison test.*

The regulation of sFLT-1 , PLGF and VEGF proteins secretion by CRH and UCN2 in HUVEC cells under high oxygen tension

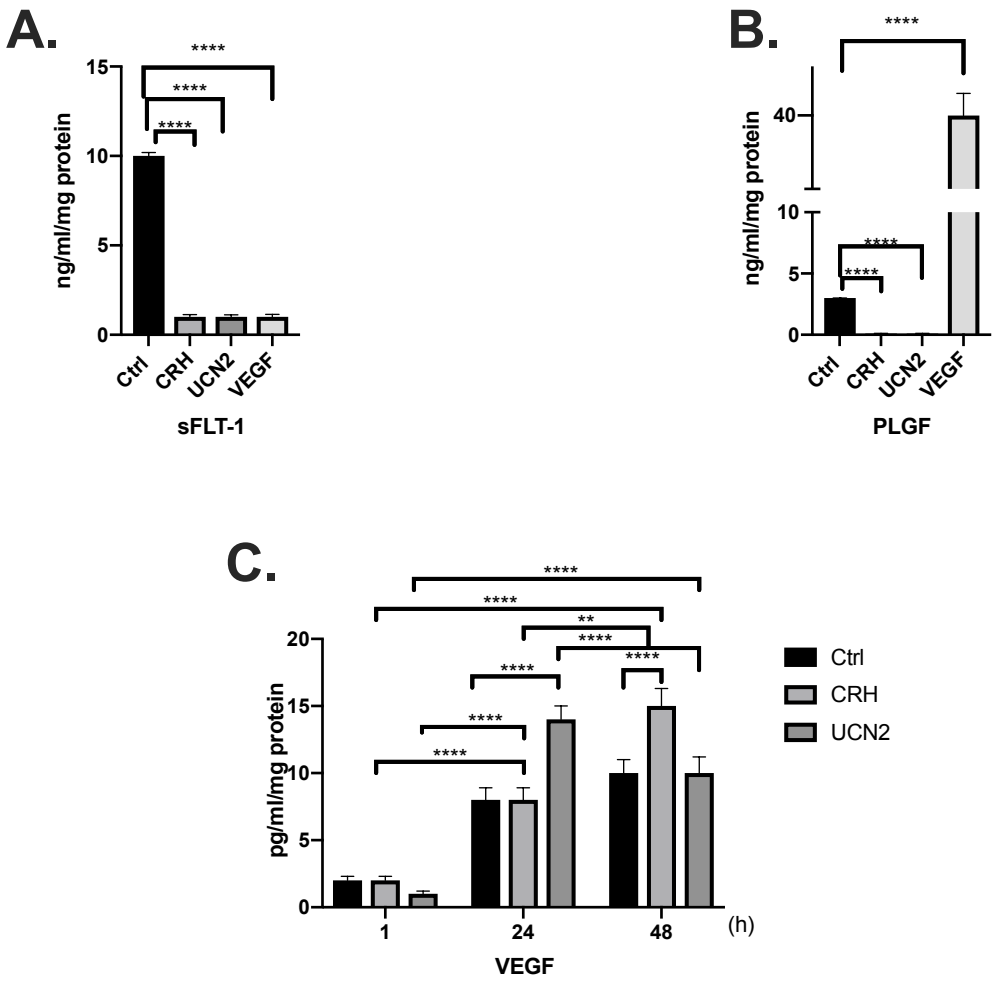


Figure 176

*CRH, UCN2 and VEGF reduces sFLT-1 protein secretion at 21% O2 at 1h in HUVEC cells. CRH, UCN2 reduces and VEGF increases PLGF protein secretion at 21% O2 at 1h in HUVEC cells. VEGF proteins were significantly released in a time dependent manner upto 48 h from HUVEC cells at 21% O2. MSD ELISA analysis showed that at 21% O2 at 1h, the protein secretion of sFLT-1 and PLGF is reduced by CRH and UCN2. The protein secretion of sFLT-1 is reduced and PLGF is increased by VEGF. CRH and UCN2 has no effect on sFLT-1 and PLGF protein secretion at 24 and 48h (data not shown). UCN2 increases protein secretion of VEGF at 24h and CRH increases VEGF release at 48h. p=0.1234 ns ; * 0.033; ** .002; *** .0002; ****< .0001; n=3.Data expressed as ng/ml normalised against mg protein. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

Table 67

CRH increases VEGF release at 48h. UCN2 increases VEGF release at 24h.

VEGF release in HUVEC	21% O2			
	Ctrl	CRH	UCN2	
1H	NS	NS	NS	
24H	NS	NS	↑	
48H	NS	↑	NS	

The regulation of VEGF mediated sFLT-1 and PLGF protein secretion by CRH at 1h in HUVEC cells under high oxygen tension

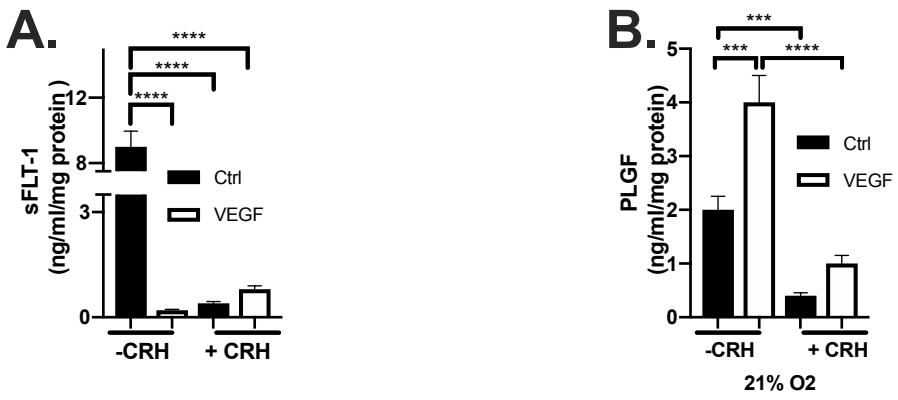


Figure 177

*CRH reverses VEGF mediated decrease in sFLT-1 and increase in PLGF protein secretion at 1h at 21% O2 in HUVEC cells. Experiment protocol, where after 80% confluency, fresh media was added and HUVEC cells were treated with 100nm CRH and 20ng/ml VEGF at 21% O2. Supernatants were collected at 1h at 21% O2 and MSD ELISA assay was done on sFLT-1 and PLGF proteins. p=*** .0002; ****< .0001; n=3.Data expressed as ng/ml normalised against mg protein. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

The regulation of sFLT-1 / PLGF ratio by CRH and VEGF at 1h in HUVEC cells under high oxygen tension

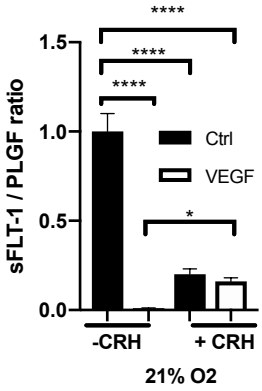


Figure 178

*VEGF and CRH reduces sFLT-1 /PLGF ratio in HUVEC cells at 1h at 21%O2. CRH reverses VEGF mediated decrease in sFLT-1 /PLGF ratio. Experiment protocol, where after 80% confluency, fresh media was added and HUVEC cells were treated with 100nm CRH and 20ng/ml VEGF at 21% O2. Supernatants were collected at 1h at 21% O2 and MSD ELISA assay was done on sFLT-1 and PLGF proteins. p=0.1234 ns ; * 0.033; ** .002; *** .0002; ****< .0001; n=3. Data expressed as ng/ml normalised against mg protein. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

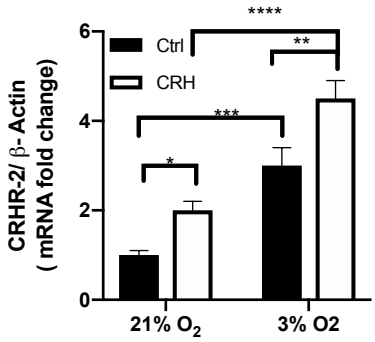


Figure 179

*Low oxygen tension upregulates mRNA expression of CRHR2 in HUVEC cells at 24h. CRH upregulates CRHR2 mRNA expression at both 21% and 3%O2. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. Cells were treated with 100nM CRH for 24h and QRT PCR analysis was done. p= * 0.033; ** .002; *** .0002; ****< .0001; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

Table 68

CRHR-1 mRNA	21% O2				3% O2			
	Ctrl	CRH	UCN2	VEGF	Ctrl	CRH	UCN2	VEGF
HUVEC	NS	NS	NS	NS	NS	NS	NS	NS
HMEC-1	NS	↓	NS	NS	↓	↑	NS	NS

HTR8 /SVneo	NS	NS	NS	NS	NS	NS	NS	NS
BEWO	NS	NS	NS	NS	↑	↓	NS	NS

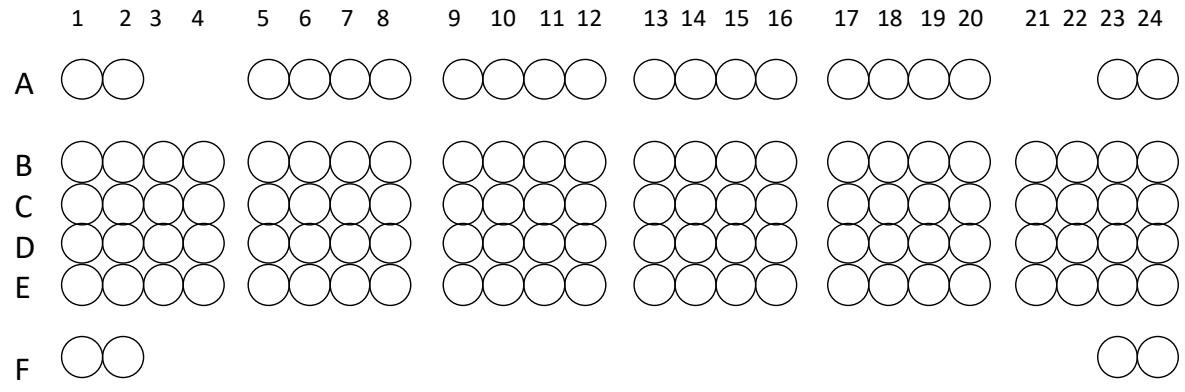
In HMEC-1 cells, CRH decreases CRHR1 mRNA expression in under high oxygen tension and this is reversed in low oxygen tension. Hypoxia reduces CRHR1 mRNA expression in HMEC-1 cells but increases in BEWO cells. In BEWO cells, in hypoxia, CRH reduces CRHR1 mRNA expression.

Table 69

CRHR-2 mRNA	**21% O2**				**3% O2**			
	Ctrl	**CRH**	**UCN2**	**VEGF**	**Ctrl**	**CRH**	**UCN2**	**VEGF**
HUVEC	NS	↑	NS	NS	↑	↑	NS	NS
HMEC-1	NS	NS	NS	NS	NS	NS	NS	NS
HTR8 /SVneo	NS	↓	NS	NS	NS	NS	NS	NS
BEWO	NS	NS	NS	NS	NS	NS	NS	NS

Hypoxia increases CRHR2 mRNA expression in HUVEC. CRH increases CRHR2 mRNA expression in HUVEC under low and high oxygen tension. Under high oxygen tension, CRH decreases CRHR2 mRNA expression in HTR8/SVneo cells.

The regulation of angiogenic machinery by CRH under high and low oxygen tension in HUVECs



Coordinate	Target/Control	Gene ID	Alternate Nomenclature
A1, A2	Reference Spots	N/A	
A5, A6	Activin A	3624	
A7, A8	ADAMTS-1	9510	
A9, A10	Angiogenin	283	ANG
A11, A12	Angiopoietin-1	284	Ang-1
A13, A14	Angiopoietin-2	285	Ang-2
A15, A16	Angiostatin/Plasmir	5340	
A17, A18	Amphiregulin	374	AR
A19, A20	Artemin	9048	
A23, A24	Reference Spots	N/A	
B1, B2	Coagulation Factor	2152	TF
B3, B4	CXCL16	58191	
B5, B6	DPPIV	1803	CD26
B7, B8	EGF	1950	
B9, B10	EG-VEGF	84432	PK1
B11, B12	Endoglin	2022	CD105
B13, B14	Endostatin/Collager	80781	
B15, B16	Endothelin-1	1906	ET-1
B17, B18	FGF acidic	2246	FGF-1
B19, B20	FGF basic	2263	FGFR2
B21, B22	FGF-4	2249	
B23, B24	FGF-7	2252	KGF
C1, C2	GDNF	2668	
C3, C4	GM-CSF	1437	
C5, C6	HB-EGF	1839	
C7, C8	HGF	3082	
C9, C10	IGFBP-1	3484	
C11, C12	IGFBP-2	3485	
C13, C14	IGFBP-3	3486	
C15, C16	IL-1β	3553	IL-1F2
C17, C18	IL-8	3576	CXCL8
C19, C20	LAP (TGF-β1)	7040	
C21, C22	Leptin	3952	
C23, C24	MCP-1	6347	CCL2
D1, D2	MIP-1α	6348	CCL3
D3, D4	MMP-8	4317	
D5, D6	MMP-9	4318	
D7, D8	NRG1-β1	3084	HRG1-β1
D9, D10	Pentraxin 3 (PTX3)	5806	TSG-14
D11, D12	PD-ECGF	1890	
D13, D14	PDGF-AA	5154	
D15, D16	PDGF-AB/PDGF-BB	5155	
D17, D18	Persephin	5623	
D19, D20	Platelet Factor 4 (PF	5196	CXCL4
D21, D22	PlGF	5228	
D23, D24	Prolactin	5617	
E1, E2	Serpin B5	5268	Maspin
E3, E4	Serpin E1	5054	PAI-1
E5, E6	Serpin F1	5176	PEDF
E7, E8	TIMP-1	7076	
E9, E10	TIMP-4	7079	
E11, E12	Thrombospondin-1	7057	TSP-1
E13, E14	Thrombospondin-2	7058	TSP-2
E15, E16	uPA	5328	
E17, E18	Vasohibin	22846	
E19, E20	VEGF	7422	
E21, E22	VEGF-C	7424	
F1, F2	Reference Spots	N/A	
F23, F24	Negative Control	N/A	Control (-)

Figure 180

Human angiogenesis array coordinates

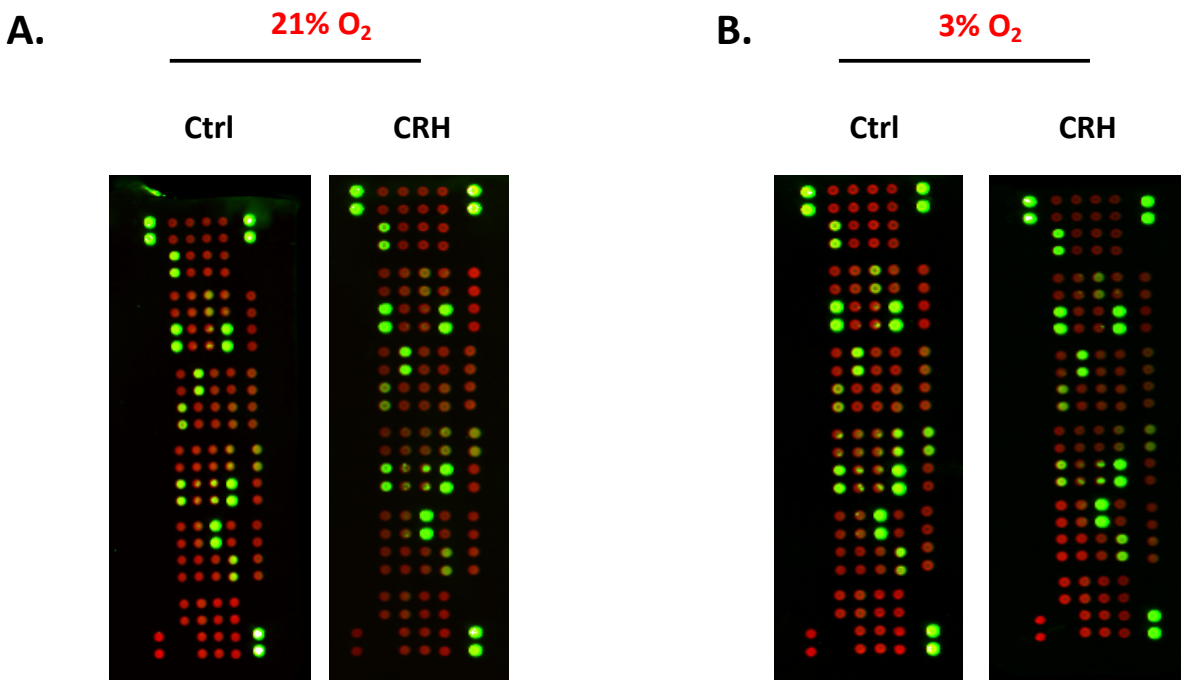


Figure 181

Angiogenesis array membranes exposed to HUVEC lysates with CRH under high (21% O2) (A) and low (3% O2) (B) oxygen tensions.

Change in Protein expression by CRH at 21% O2	
Angiopoietin 2	↑
EGF	↓
Endostatin	↑
Serpin E1	↑
PDGFAB/BB	↑
IL1B	↑
IGF-BP3	↑
GM-CSF	↓
UPA /PLAU	↑
VEGF	↑

Change in Protein expression by CRH at 3% O2	
Angiogenin	↓
Endoglin	↓
Endostatin	↓
Endothelin 1	↓
Serpin E1	↑
TIMP-1	↓
IL - 8	↑
PDGFAA	↓
IL1B	↑
IGF-BP3	↓
HB-EGF	↓
FGF-Basic	↑
UPA / PLAU	↓
HGF	↓
angiopoietin 2	↓
Amphiregulin	↓
CXCL16	↓
DPP IV	↓
Thrombospondin 2	↓

Figure 182

Common angiogenic molecules regulated by CRH at both oxygen tension.Angiogenesis array analysis (LICOR) showed that the under high oxygen tension, CRH increases the protein expression of Angiopoietin 2, Endostatin, Serpin E1, PDGFAB/BB, IL-1B, IGF-BP3, UPA/PLAU, VEGF and decreases the expression of EGF, GM-CSF . Under low oxygen tension, CRH decreases protein expression of angiogenin, endoglin, endostatin, endothelin 1, TIMP-1, IL-8, PDGF-AA, IGF-BP3, HB-EGF, UPA/PLAU, HGF, angiopoietin 2, amphiregulin, CXCL-16, DPP IV, thrombospondin 2 and increases expression of IL-1B, FGF- Basic and serpin E1.

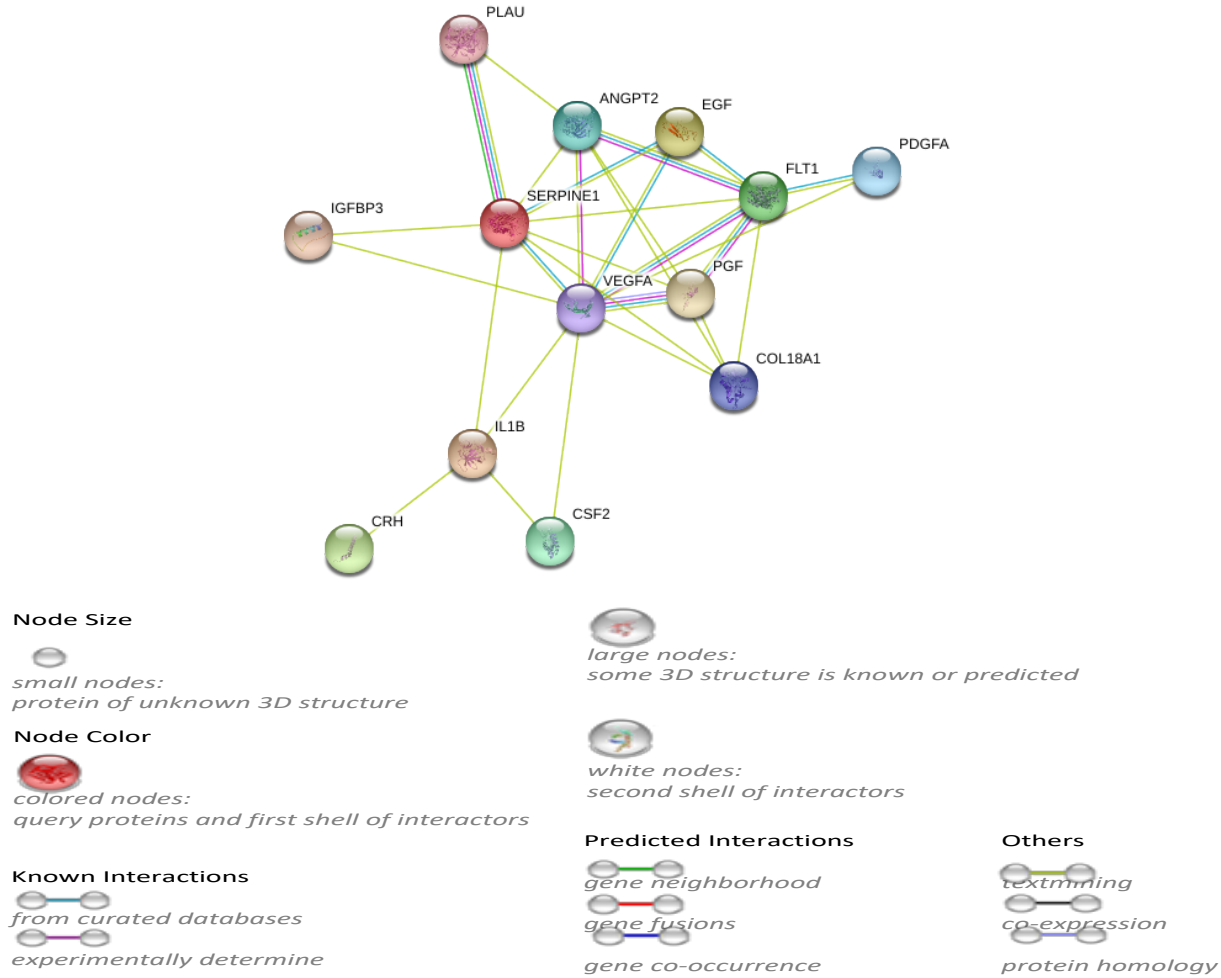


Figure 183

pictorial representation of protein network of angiogenic molecules that are possibly regulated by CRH under high oxygen tension in HUVEC (String software analysis). Node size : Small nodes - proteins of unknown 3D structure; large nodes - some 3D structure is known or predicted. Node color: colored nodes - query proteins and first shell of interactors; white nodes - second shell of interactors. Known interactions : blue lines - from curated databases; pink lines - experimentally determine. Predicted interactions : gene neighborhood - green lines; gene fusions - red lines, gene co-occurrence - blue lines.

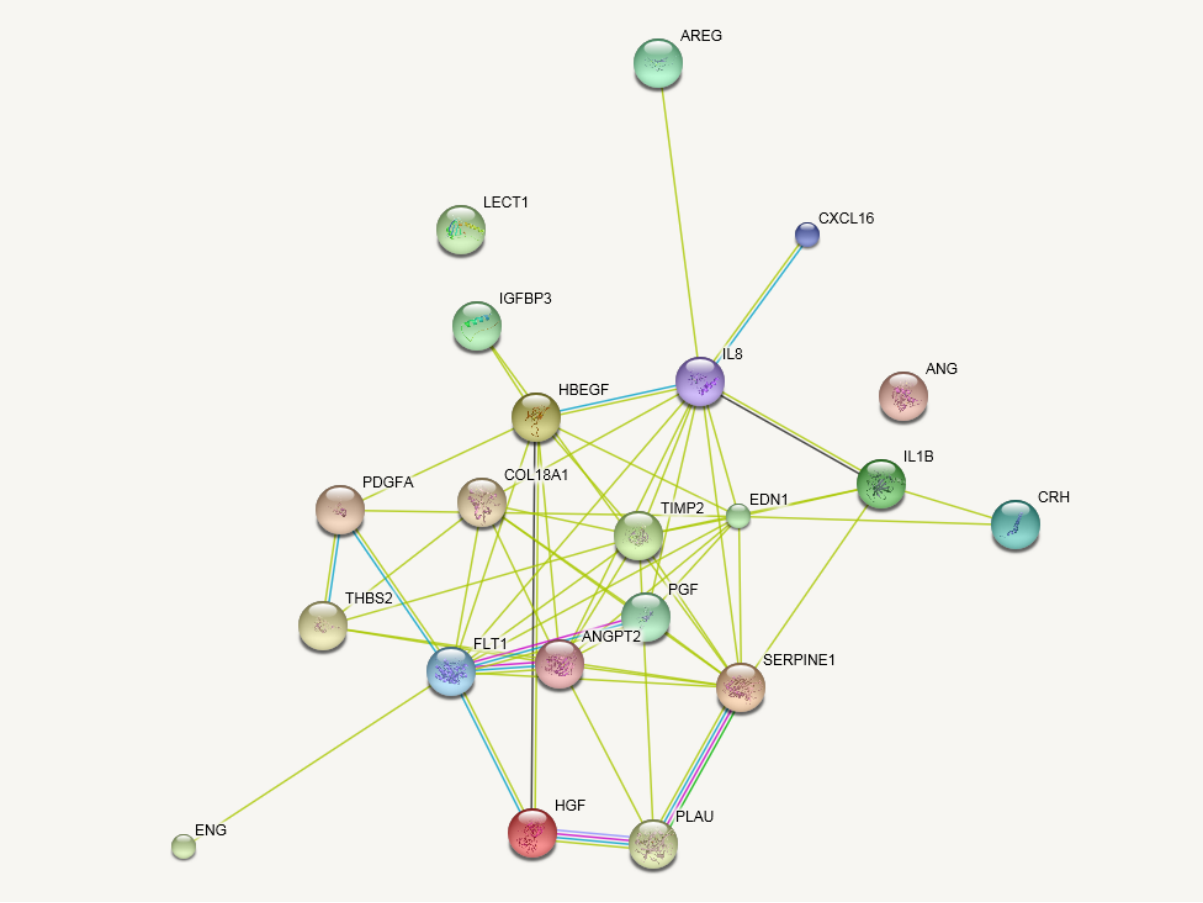


Figure 184

pictorial representation of protein network of angiogenic molecules that are possibly regulated by CRH under low oxygen tension in HUVEC (String software analysis).

Table 70

	21% O2	3% O2
Angiopoietin 2	↑	↓
Endostatin	↑	↓
Serpin E1	↑	↑
PDGFAB/BB	↑	↓
IL1B	↑	↑
IGF-BP3	↑	↓
UPA /PLAU	↑	↓

Low oxygen tension reverses the upregulation of angiogenic molecules – Angiopoietin 2, Endostatin, PDGFAB/BB, IGF-BP3 and UPA/PLAU, Except Serpin E1 and IL1B (blue), all the angiogenic molecules that were upregulated in normoxia was downregulated in hypoxia.

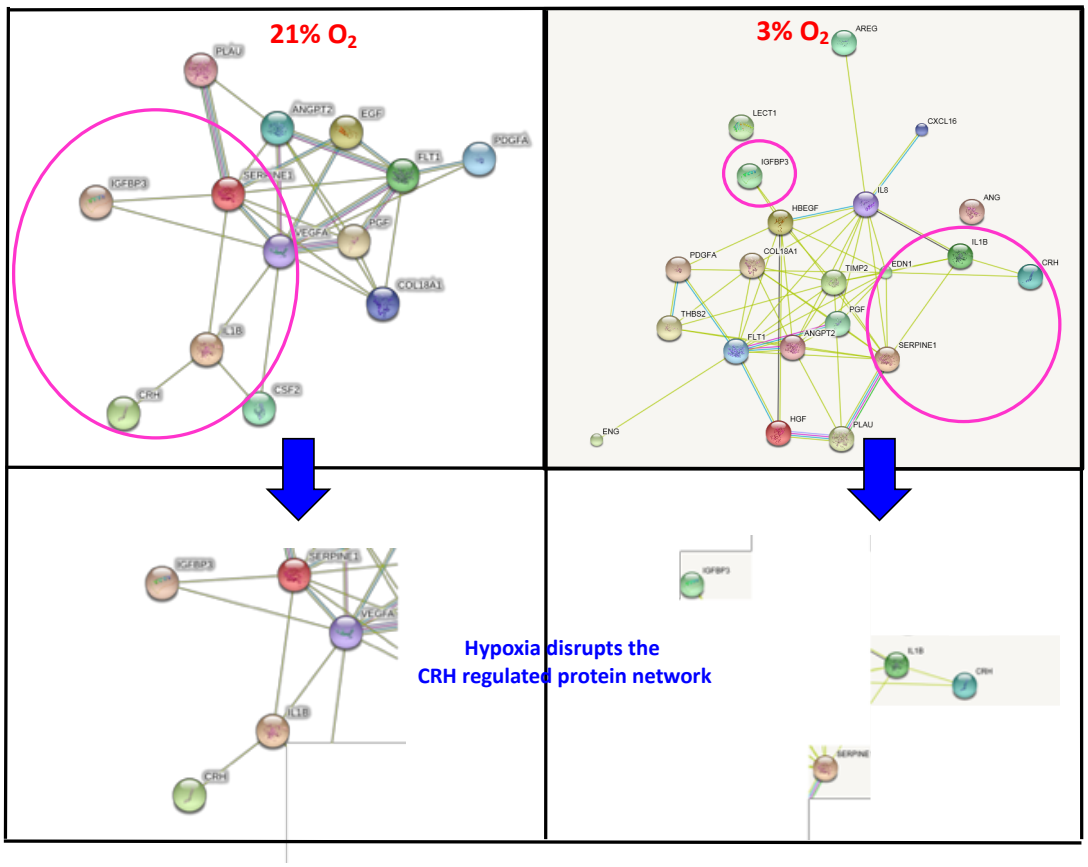


Figure 185

Investigation of interactions or enabled formation of interactive network of angiogenic molecules by String software analysis showed hypoxia disrupts the regulation of angiogenic protein network by CRH. Possibly new nodes might be formed or new linkages amongst the nodes might be established under hypoxic condition.

Huvec 15'

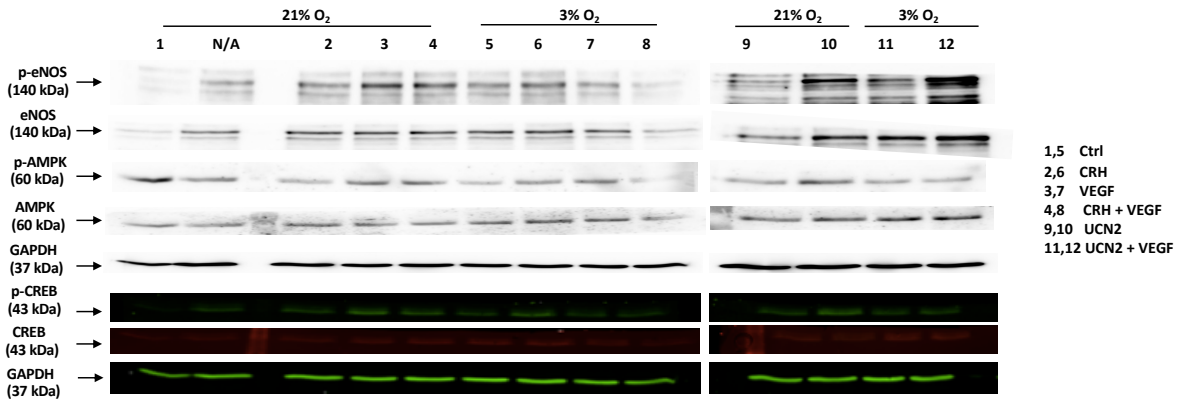


Figure 186

Huvec 60’

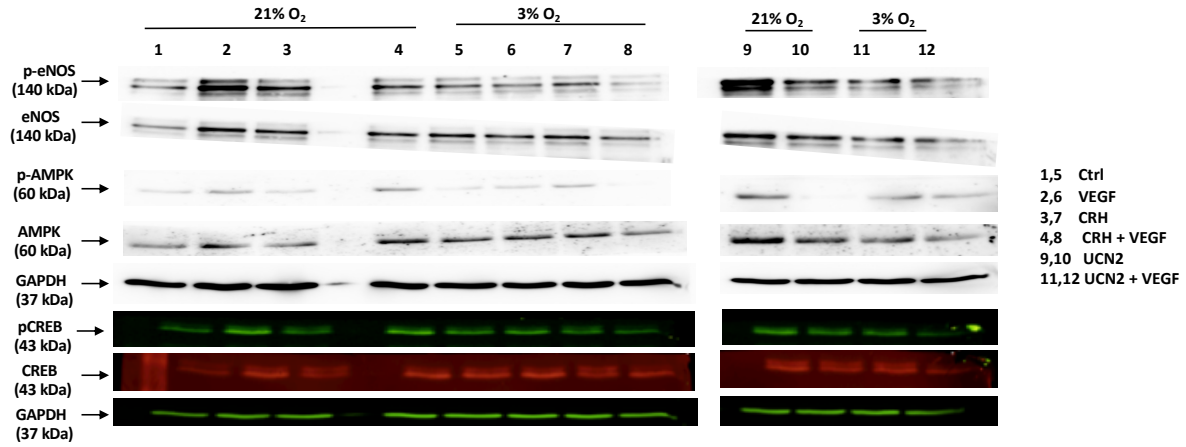


Figure 187

Huvec 24h

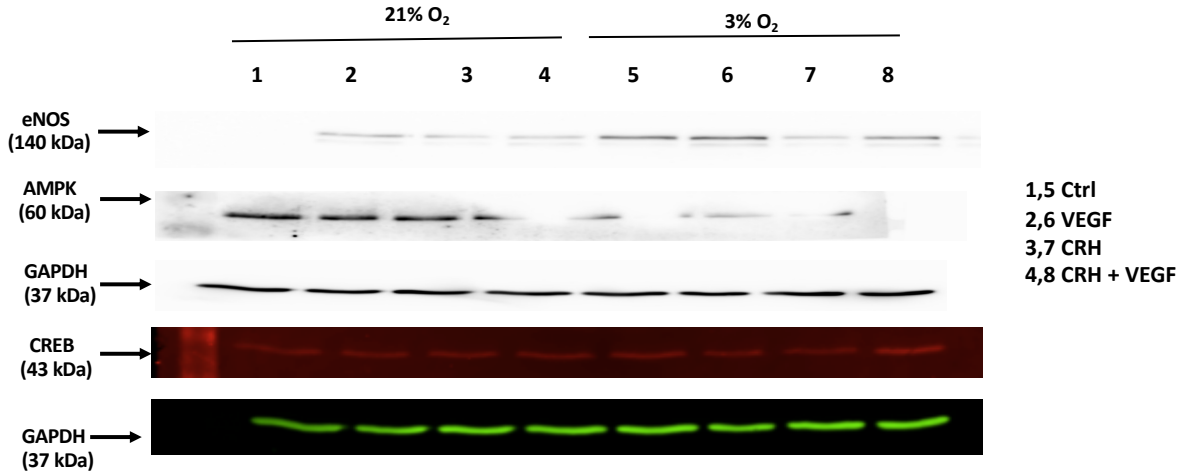


Figure 188

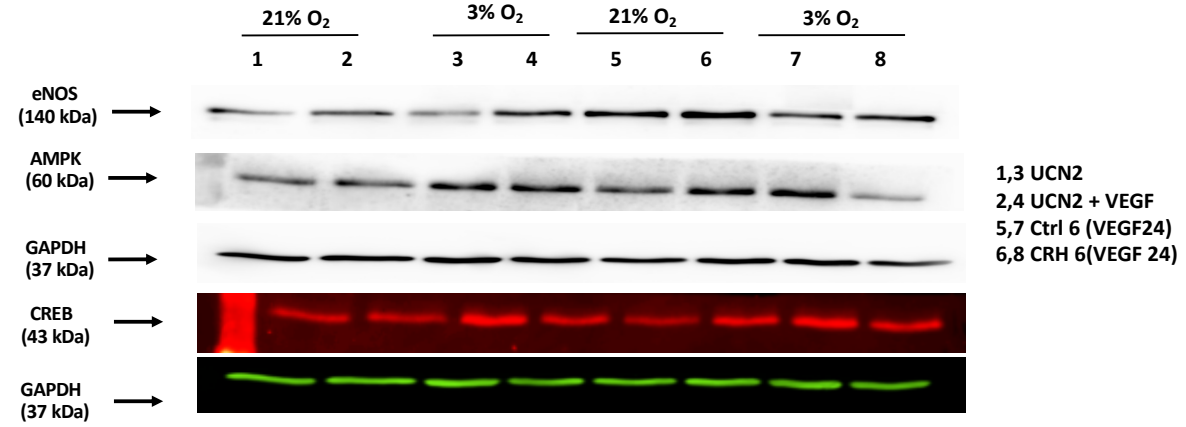


Figure 189

HUVEC

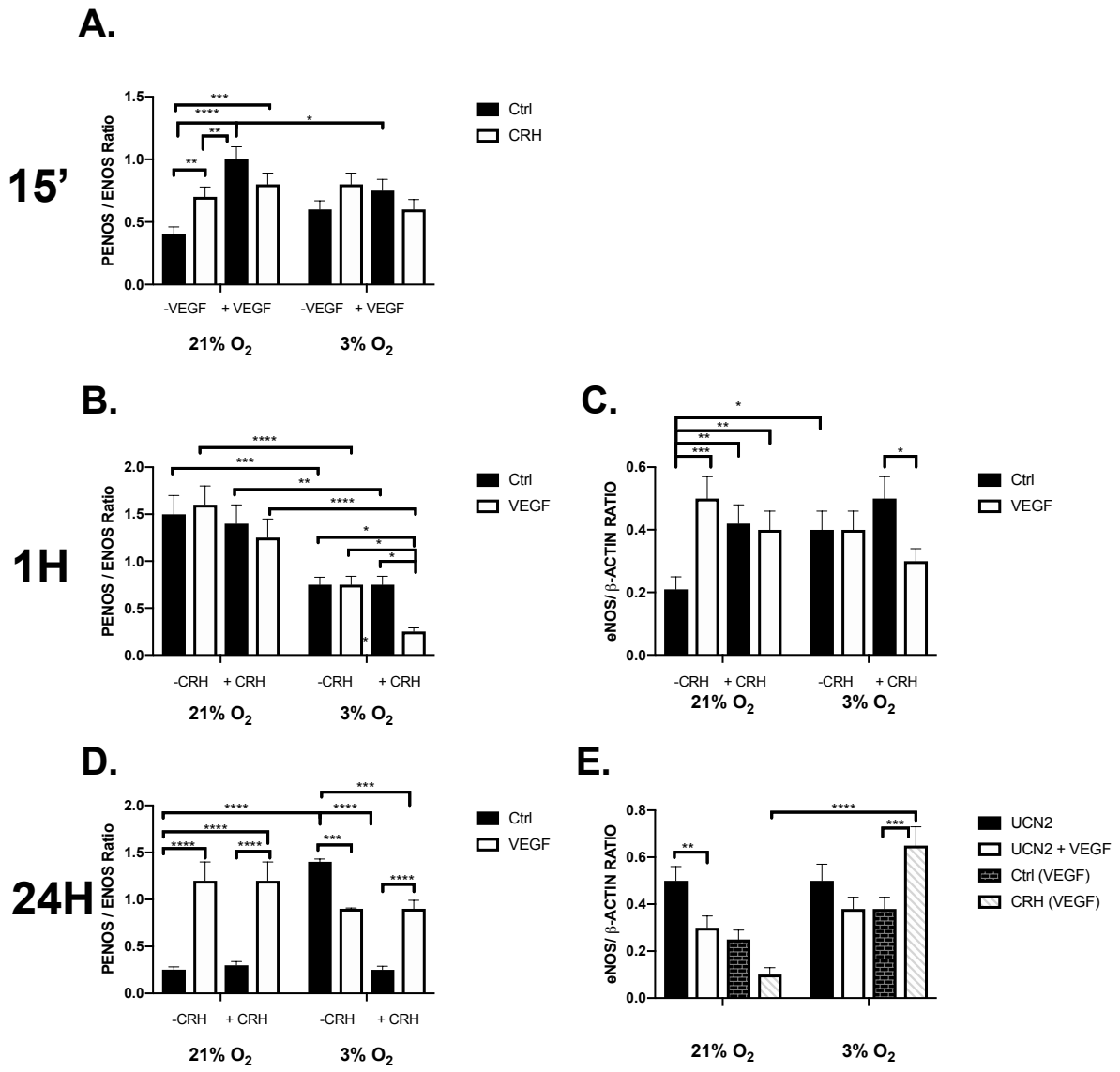


Figure 190

VEGF increases pENOS.CRH increases pENOS.

Table 71

p-eNOS /eNOS ratio	21% O2				3% O2			
	Ctrl	CRH	VEGF	CRH+VEGF	Ctrl	CRH	VEGF	CRH+VEGF
15'	NS	↑	↑	NS	NS	NS	NS	NS
1H	NS	NS	NS	NS	↓	NS	NS	↓

24H	NS	NS	↑	↑	↑	↓	↓	↑								

CRH increases phosphorylation of eNOS at 15'. VEGF increases phosphorylation of eNOS at 15', transiently lost at 1h, which is further increased at 24h. Hypoxia abolishes the CRH and VEGF stimulated increase in phosphorylation of eNOS at 15'. CRH has no effect on VEGF induced phosphorylation of eNOS at 24h. Hypoxia increases phosphorylation of eNOS at 24h and reverses VEGF induced increase in phosphorylation of eNOS. CRH reduces pENOS at 24h in hypoxia. CRH has no effect on VEGF mediated phosphorylation of ENOS under normal and low oxygen tension.

Table 72

eNOS / GAPDH ratio	**21% O2**								**3 % O2**							
	Ctrl	**CRH**	**VEGF**	**CRH+ VEGF**	**UCN2**	**UCN2 + VEGF**	**Ctrl (VEGF)**	**CRH (VEGF)**	**Ctrl**	**CRH**	**VEGF**	**CRH+ VEGF**	**UCN2**	**UCN2 + VEGF**	**Ctrl (VEGF)**	**CRH (VEGF)**
1H	NS	↑	↑	NS	NS	NS	NS	NS	↑	NS	NS	↓	NS	NS	NS	NS
24H	NS	↑	NS	↑	NS	↓	NS	NS	↑	↓	↓	↑	NS	NS	NS	↑

CRH increases eNOS protein expression at 1h and 24h.

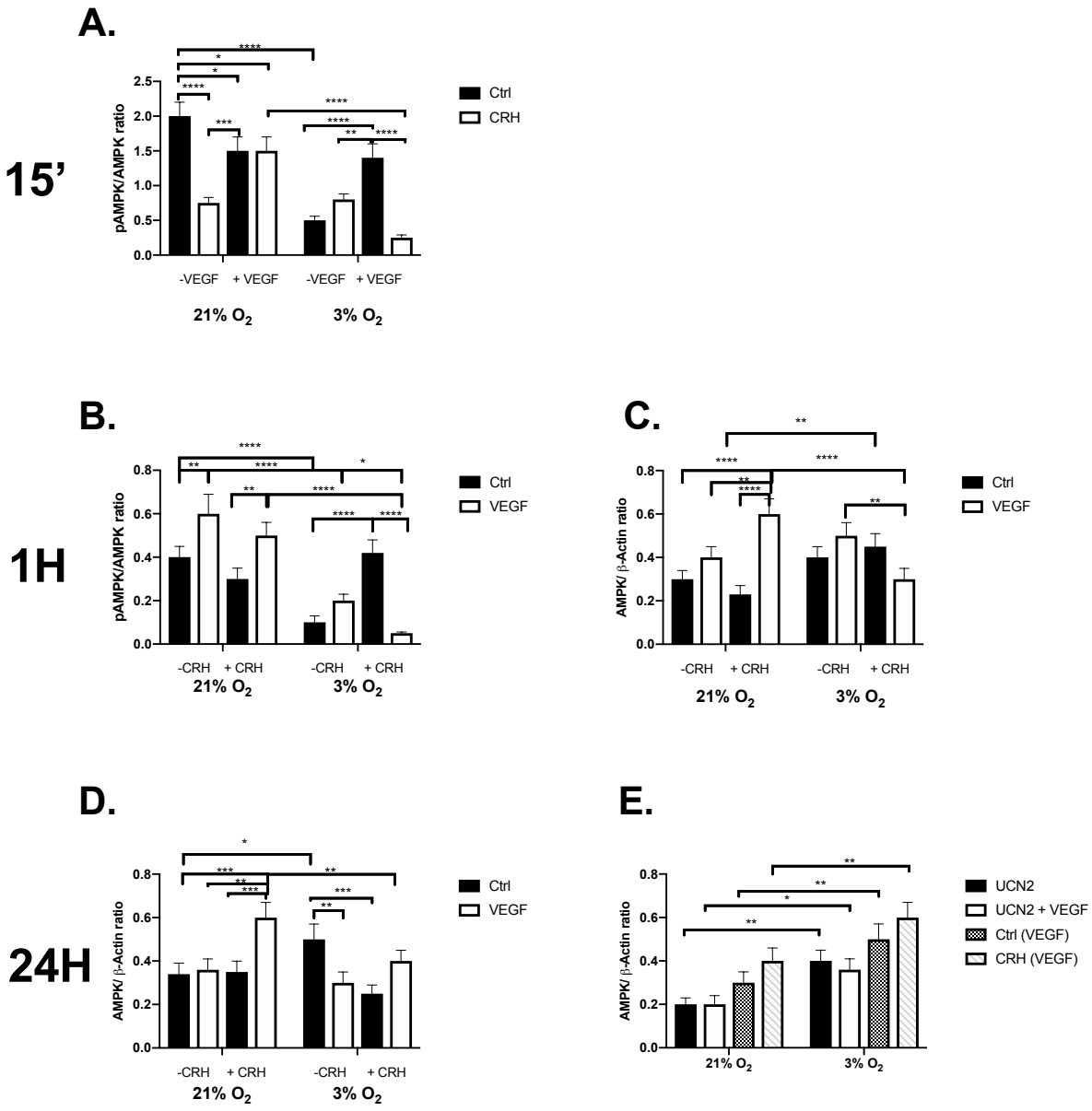


Figure 191

Table 73

p-AMPK/AMPK ratio	21% O2				3% O2			
	Ctrl	CRH	VEGF	CRH+VEGF	Ctrl	CRH	VEGF	CRH+VEGF
15’	NS	↓	↓	↓	↓	NS	↑	↓
1H	NS	↑	NS	↑	↓	NS	↑	↓
24H	NS	NS	NS	↑	↑	↓	↓	↓

Table 74

AMPK/ GAPDH ratio	21% O2								3 % O2							
	Ctrl	CRH	VEGF	CRH+ VEGF	UCN2	UCN2 + VEGF	Ctrl (VEGF)	CRH (VEGF)	Ctrl	CRH	VEGF	CRH+ VEGF	UCN2	UCN2 + VEGF	Ctrl (VEGF)	CRH (VEGF)
1H	NS	NS	NS	↑	NS	NS	NS	NS	NS	NS	NS	↓	NS	NS	NS	NS
24H	NS	NS	NS	↑	NS	NS	NS	NS	↑	↓	↓	NS	NS	NS	NS	NS

Huvec 15’

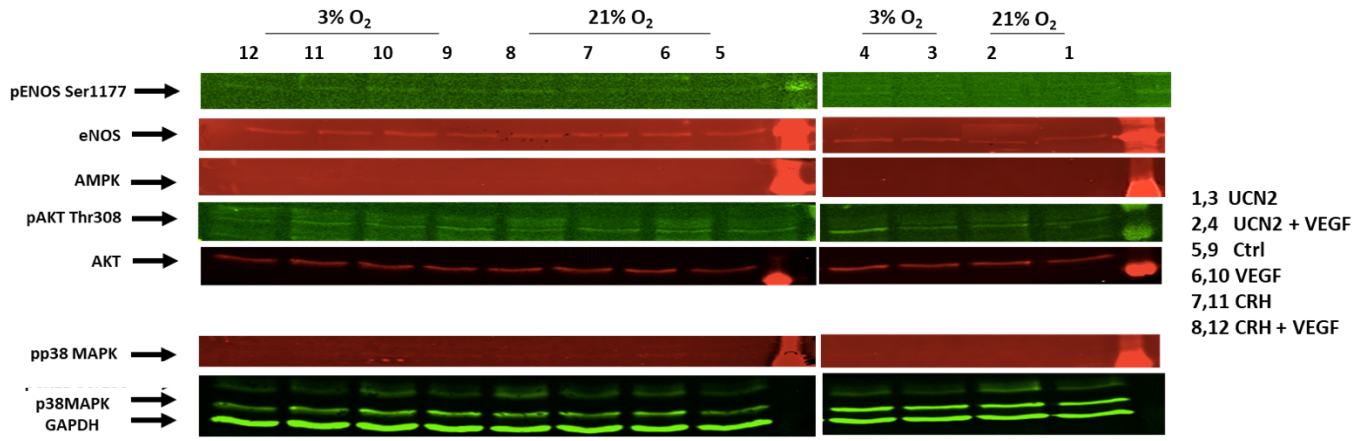


Figure 192

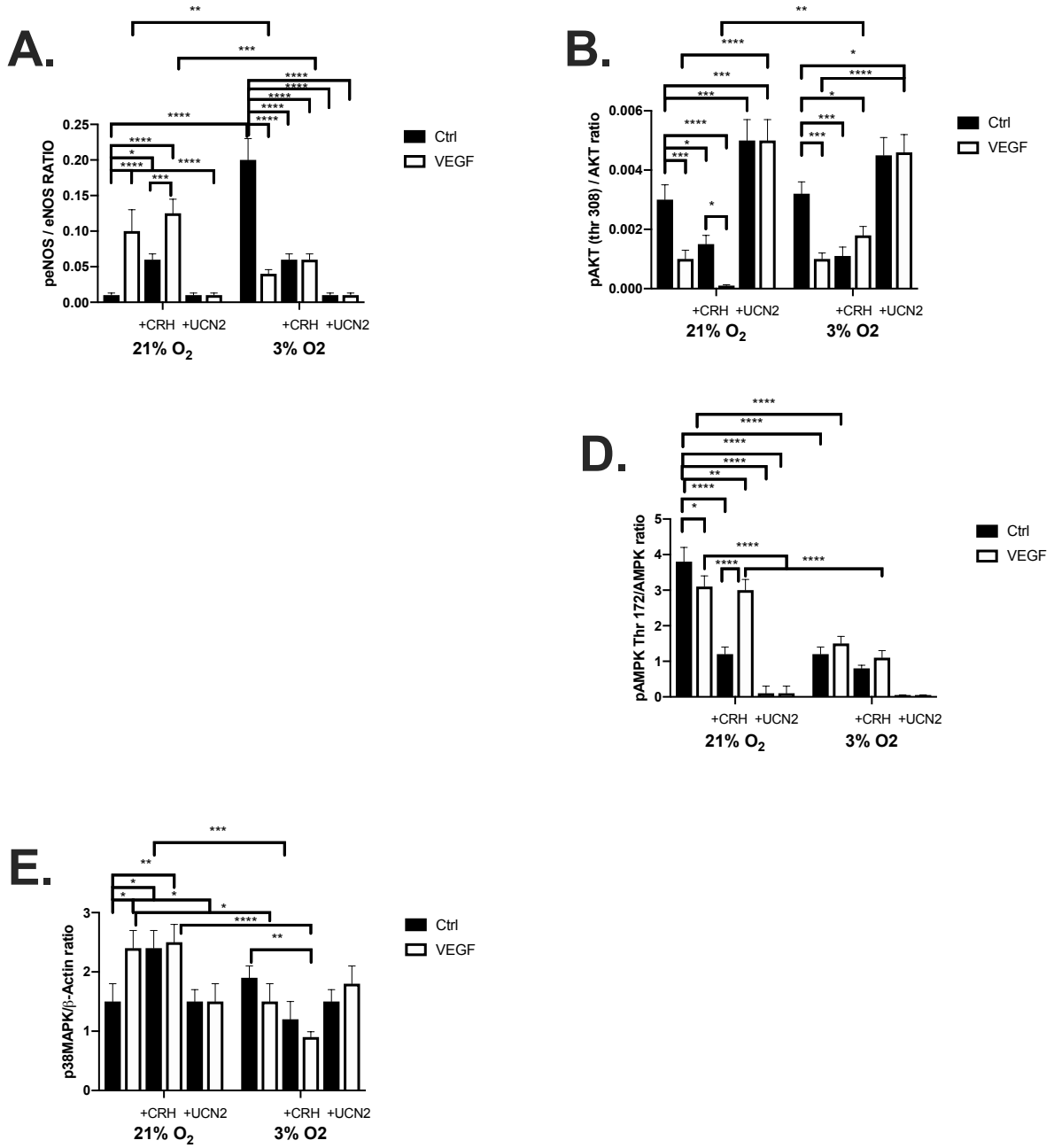


Figure 193

Table 75

15'	21 % O2						3% O2					
	Ctrl	CRH	VEGF	CRH +VEGF	UCN2	UCN2 + VEGF	Ctrl	CRH	VEGF	CRH +VEGF	UCN2	UCN2+ VEGF
pENOS/eNOS ratio	NS	↑	↑	↑	NS	NS	↑	↓	↓	NS	↓	NS

pAKT thr308 /AKT ratio	NS	↓	↓	↓	↑	NS	NS	↓	↓	NS	↑	NS
pAMPK /AMPK ratio	NS	↓	↓	↑	↓	NS	↓	NS	NS	NS	↓	NS
P38 MAPK /GAPDH ratio	NS	↑	↑	NS	NS	NS	NS	NS	NS	NS	NS	NS

HUVEC 1H

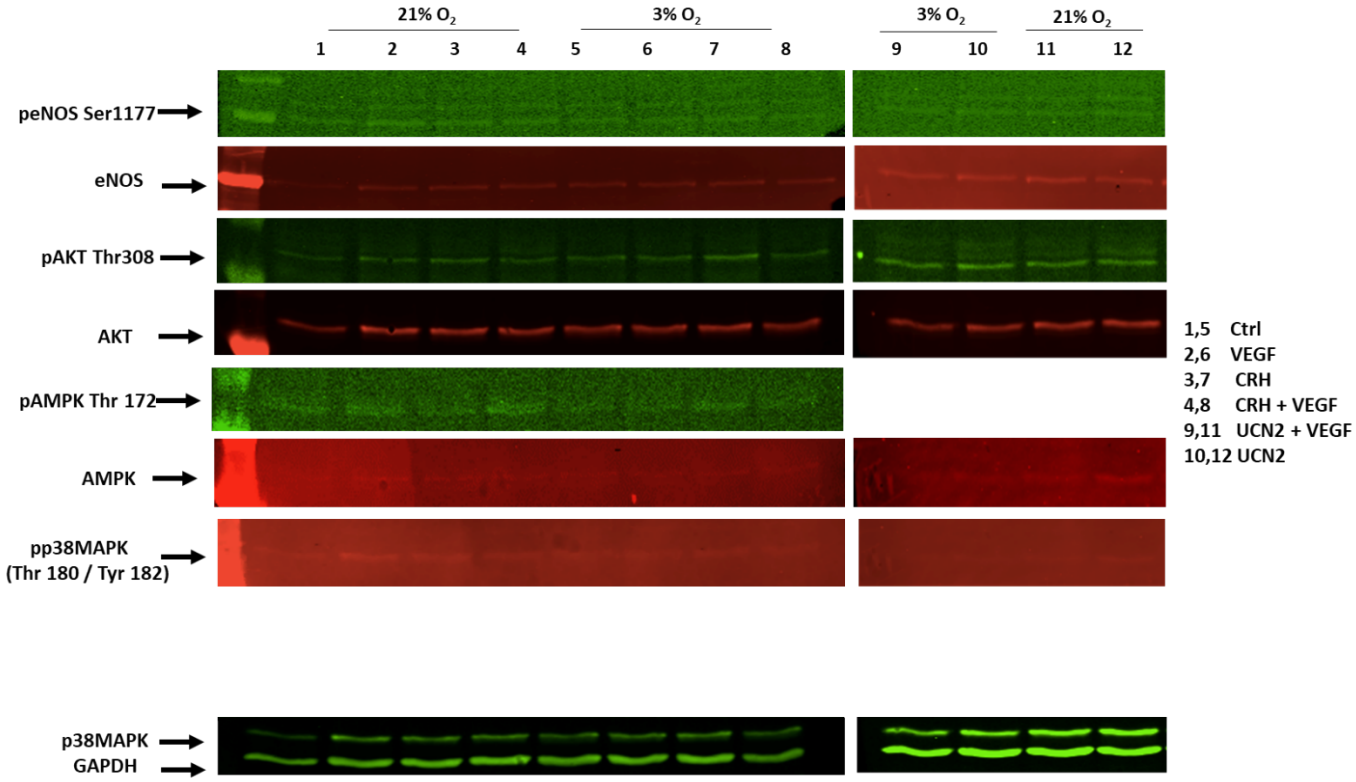


Figure 194

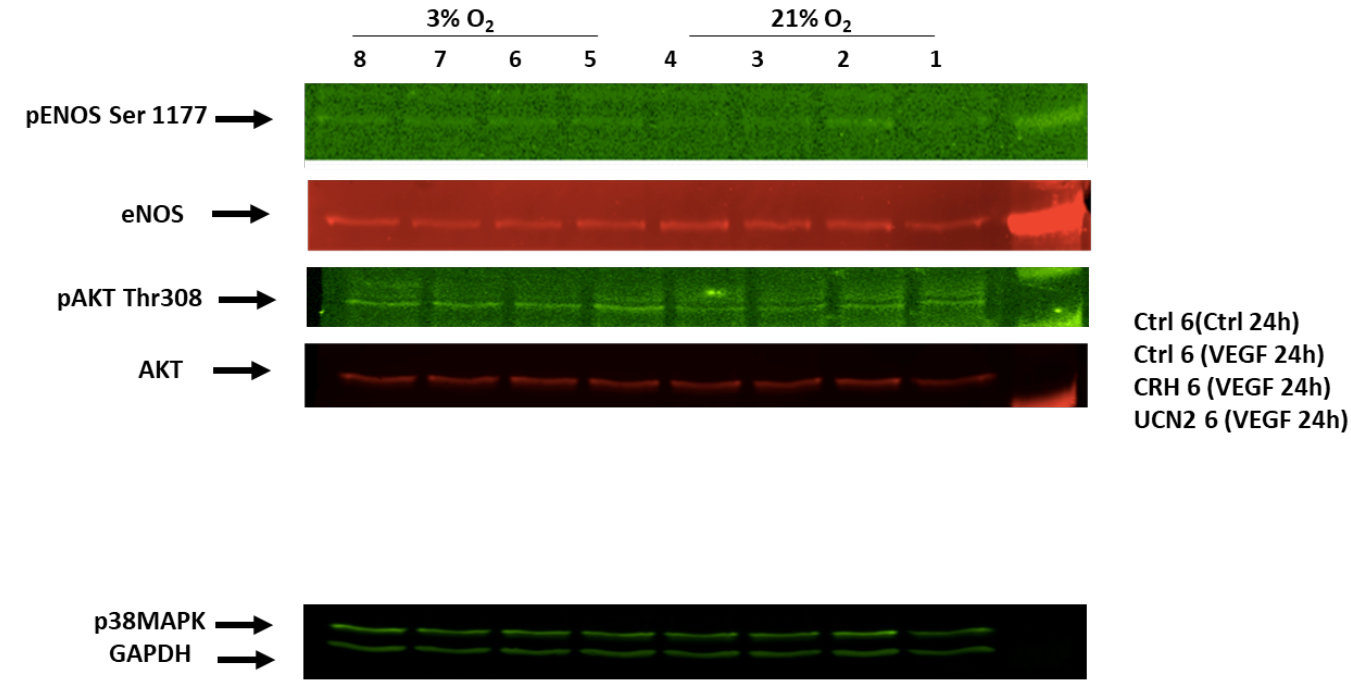


Figure 195

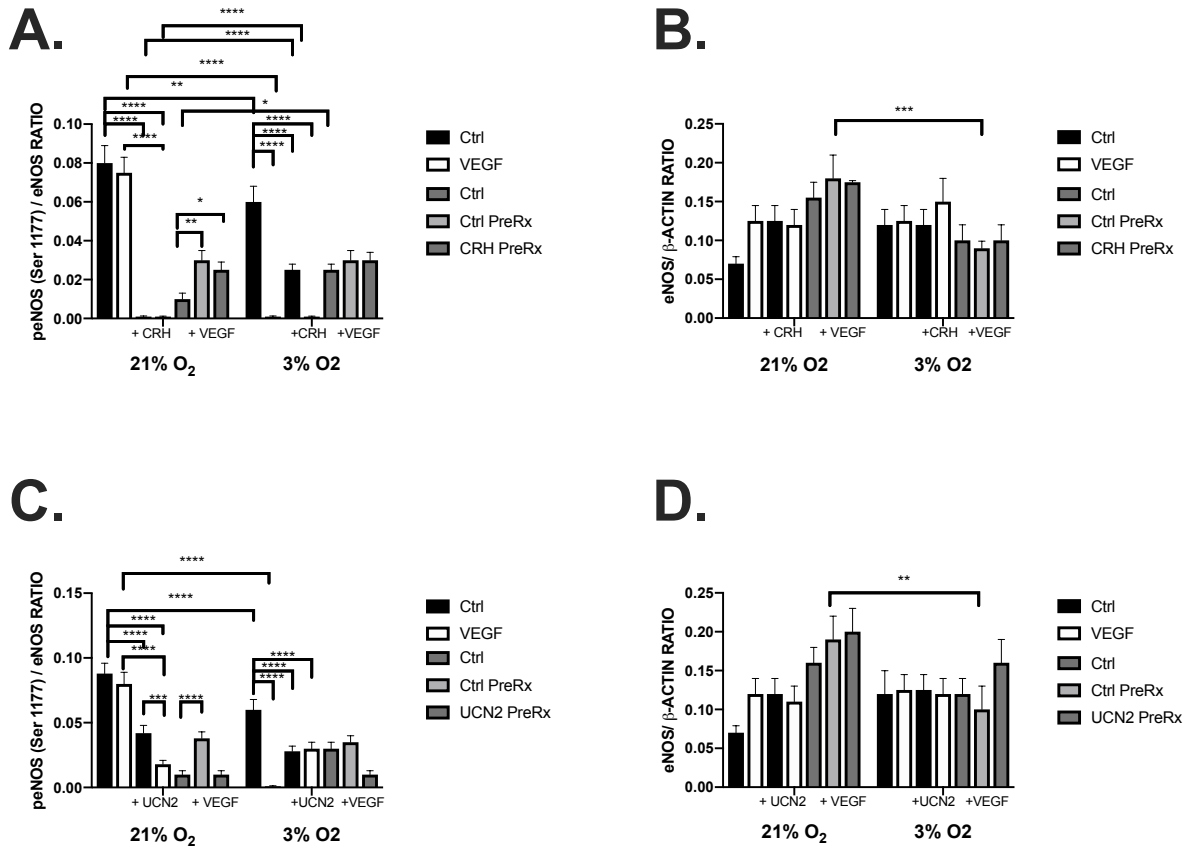


Figure 196

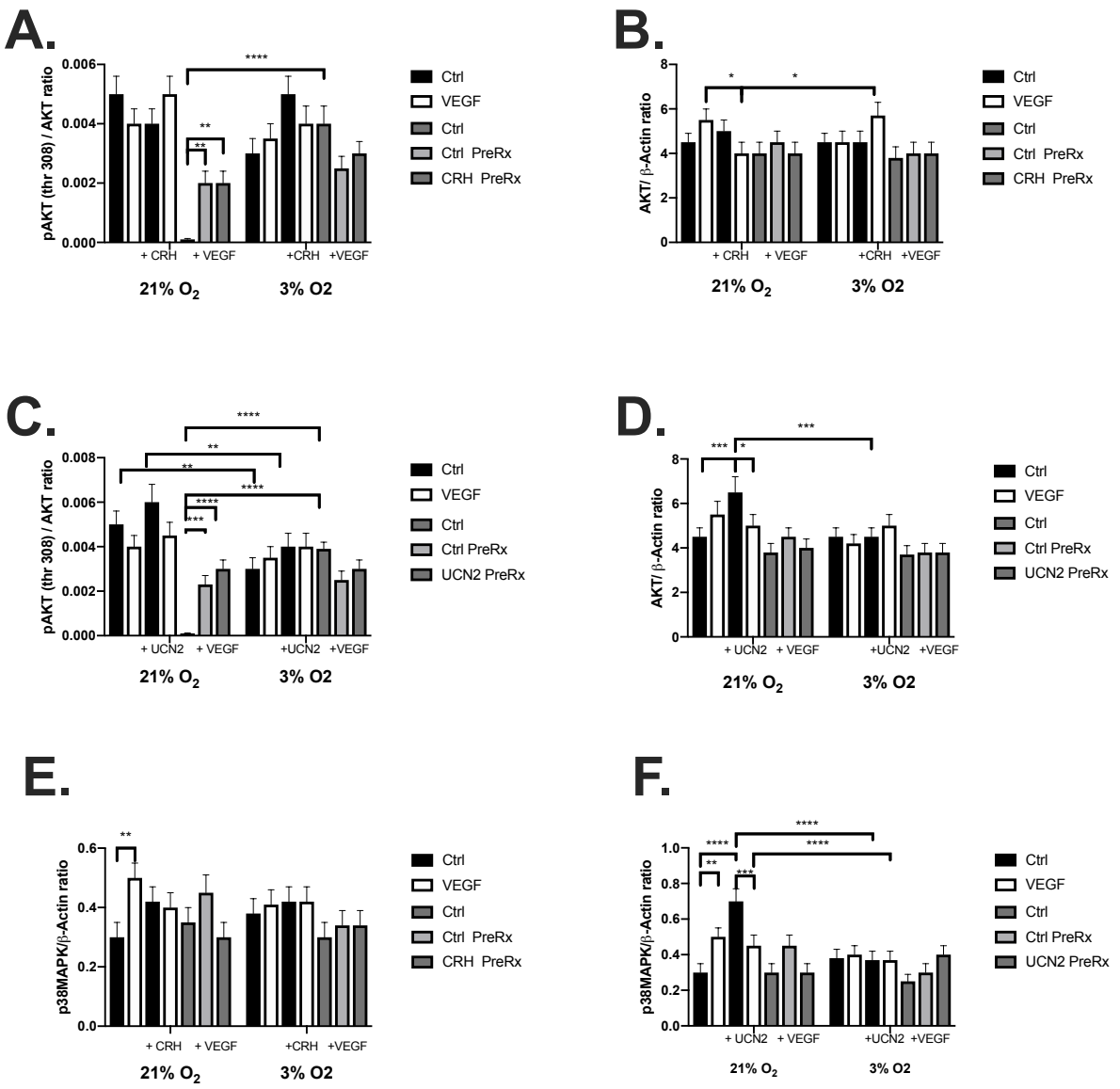

A.
pAKT (thr 308) / AKT ratio
0.006
0.004
0.002
0.000
****
**
**
+ CRH
+ VEGF
+CRH
+VEGF
21% O2
3% O2
Ctrl
VEGF
Ctrl
Ctrl PreRx
CRH PreRx
B.
AKT/ β-Actin ratio
8
6
4
2
0
*
*
+ CRH
+ VEGF
+CRH
+VEGF
21% O2
3% O2
Ctrl
VEGF
Ctrl
Ctrl PreRx
CRH PreRx
C.
pAKT (thr 308) / AKT ratio
0.008
0.006
0.004
0.002
0.000
****
**
**
****
****
***
+ UCN2
+ VEGF
+UCN2
+VEGF
21% O2
3% O2
Ctrl
VEGF
Ctrl
Ctrl PreRx
UCN2 PreRx
D.
AKT/ β-Actin ratio
8
6
4
2
0
***
*
***
+ UCN2
+ VEGF
+UCN2
+VEGF
21% O2
3% O2
Ctrl
VEGF
Ctrl
Ctrl PreRx
UCN2 PreRx
E.
p38MAPK/β-Actin ratio
0.6
0.4
0.2
0.0
**
+ CRH
+ VEGF
+CRH
+VEGF
21% O2
3% O2
Ctrl
VEGF
Ctrl
Ctrl PreRx
CRH PreRx
F.
p38MAPK/β-Actin ratio
1.0
0.8
0.6
0.4
0.2
0.0
****
****
**
***
+ UCN2
+ VEGF
+UCN2
+VEGF
21% O2
3% O2
Ctrl
VEGF
Ctrl
Ctrl PreRx
UCN2 PreRx

Figure 197

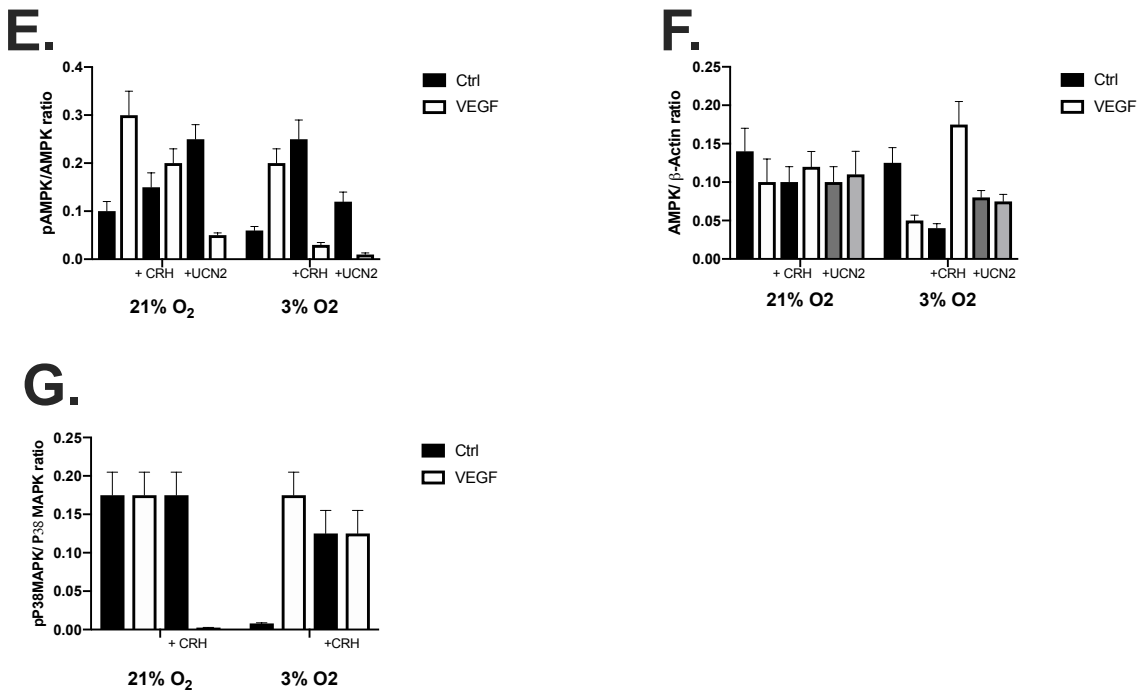


Figure 198

Table 76

1H	21% O2						3% O2					
	Ctrl	VEGF	CRH	CRH +VEGF	UCN2	UCN2+VEGF	Ctrl	VEGF	CRH	CRH +VEGF	UCN2	UCN2+VEGF
pENOS/eNOS ratio	NS	NS	↓	NS	↓	↓	↓	↓	↓	↓	↓	NS
pAKT thr308 /AKT ratio	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
AKT/ GAPDH ratio	NS	NS	NS	NS	↑	↓	NS	NS	NS	NS	NS	NS
pAMPK /AMPK ratio	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P38 MAPK /GAPDH ratio	NS	↑	NS	NS	↑	↓	NS	NS	NS	NS	NS	NS
PAMPK/AMPK ratio	NS	↑	NS	NS	↑	↓	NS	NS	NS	NS	NS	NS

AMPK /GAPDH ratio	NS	NS	NS	NS	NS	NS	NS	↓	↓	↑	↓	NS
P38 MAPK/GAPDH ratio	NS	NS	NS	NS	NS	NS	↓	↑	NS	NS	NS	NS

Huvec 24h

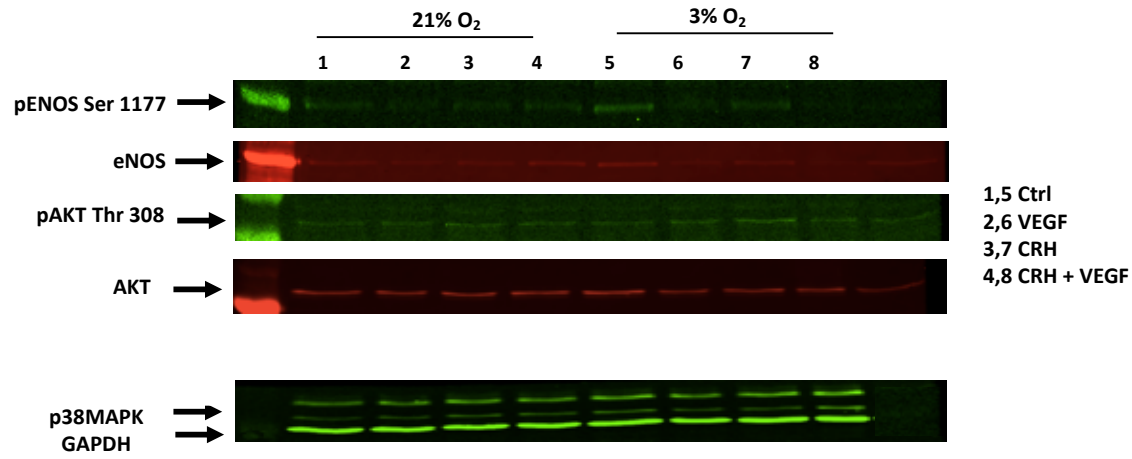


Figure 199

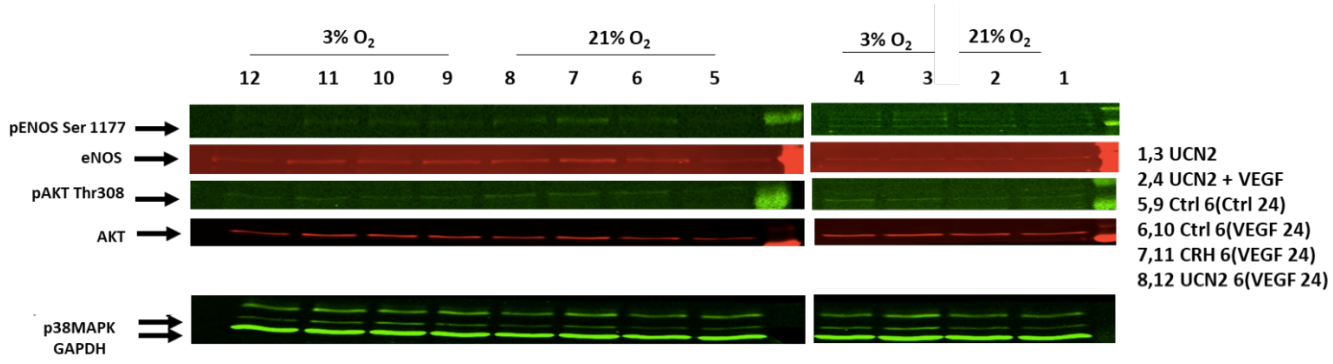


Figure 200

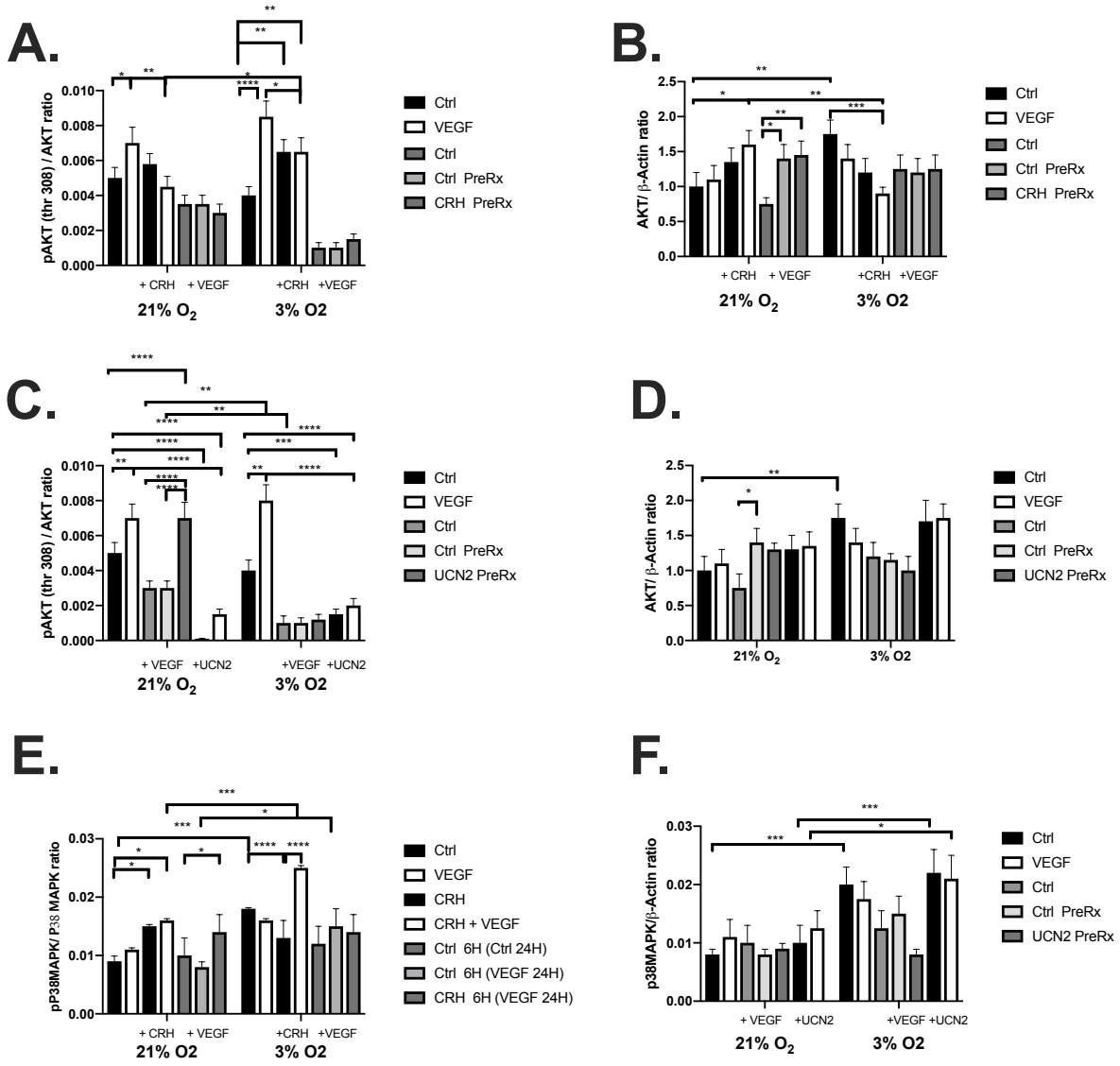


Figure 201

Table 77

24H	21 % O2									3% O2								
	Ctrl	VEGF	CRH	CRH +VEGF	Ctrl (VEGF)	CRH (VEGF)	UCN2 (VEGF)	UCN2	UCN2 +VEGF	Ctrl	VEGF	CRH	CRH +VEGF	Ctrl (VEGF)	CRH (VEGF)	UCN2 (VEGF)	UCN2	UCN2 +VEGF
PENOS/ENOS ratio	NS	↓	↓	NS	NS	↑	NS	NS	↓	NS	NS	↓	↓	NS	↓	NS	NS	↓
ENOS/ GAPDH ratio	NS	NS	NS	↑	NS	NS	NS	NS	↓	↑	↓	↓	↑	NS	NS	NS	↓	NS
PAKT Thr 308 /AKT ratio	NS	↑	NS	↓	NS	NS	↑	↓	↓	NS	↑	NS	↓	NS	NS	NS	↓	↓

AKT /GAPDH ratio	NS	NS	NS	NS	NS	NS	NS	NS	NS	↑	NS	NS	NS	NS	NS	NS	NS	NS
P38 MAPK / P38 MAPK ratio	NS	NS	↑	NS	NS	NS	NS	NS	NS	↑	NS	↓	↑	NS	NS	NS	NS	NS
P38 MAPK / GAPDH ratio	NS	NS	NS	NS	NS	NS	NS	NS	NS	↑	NS	NS	NS	NS	NS	NS	NS	NS

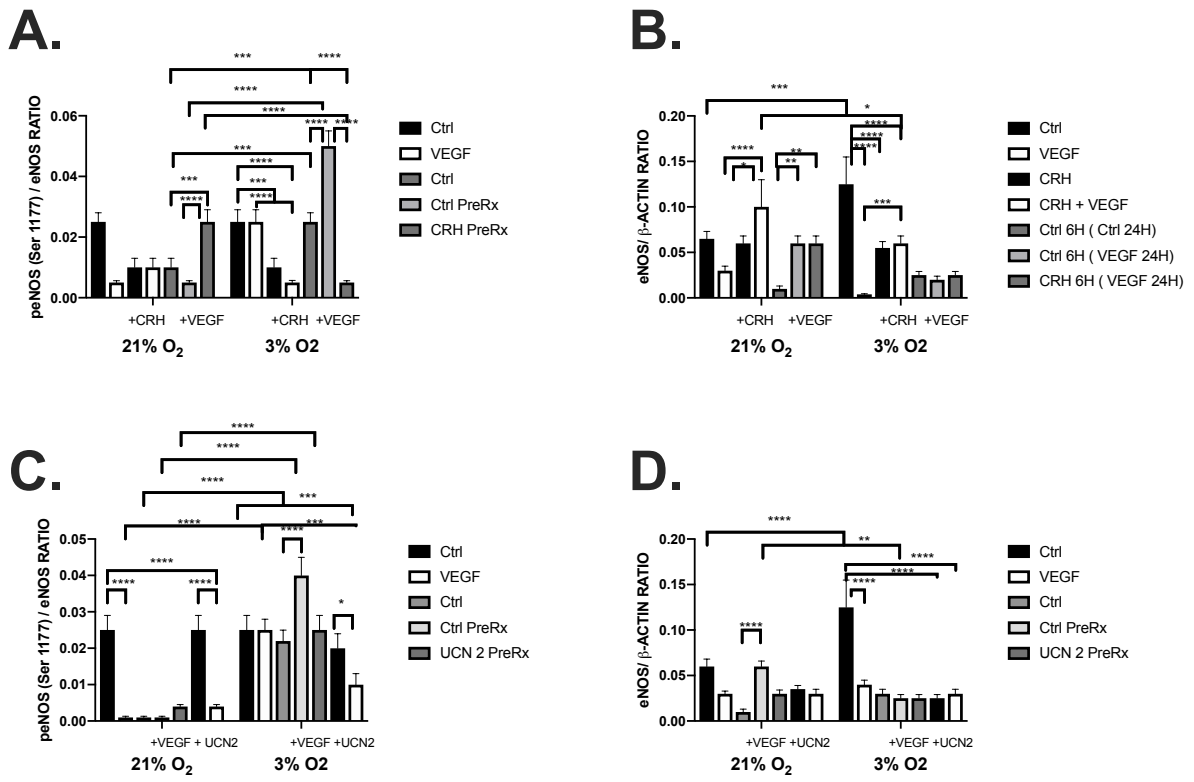


Figure 202

Table 78

24H	**21 % O2**									**3% O2**								
	Ctrl	**VEGF**	**CRH**	**CRH +VEGF**	**Ctrl (VEGF)**	**CRH (VEGF)**	**UCN2 (VEGF)**	**UCN2**	**UCN2 +VEGF**	**Ctrl**	**VEGF**	**CRH**	**CRH +VEGF**	**Ctrl (VEGF)**	**CRH (VEGF)**	**UCN2 (VEGF)**	**UCN2**	**UCN2 +VEGF**
PENOS/ENOS ratio	NS	↓	↓	NS	NS	↑	NS	NS	↓	NS	NS	↓	↓	NS	↓	NS	NS	↓
ENOS/ GAPDH ratio	NS	NS	NS	↑	NS	NS	NS	NS	NS	↑	↓	↓	↑	NS	NS	NS	↓	NS

6.4 HMEC

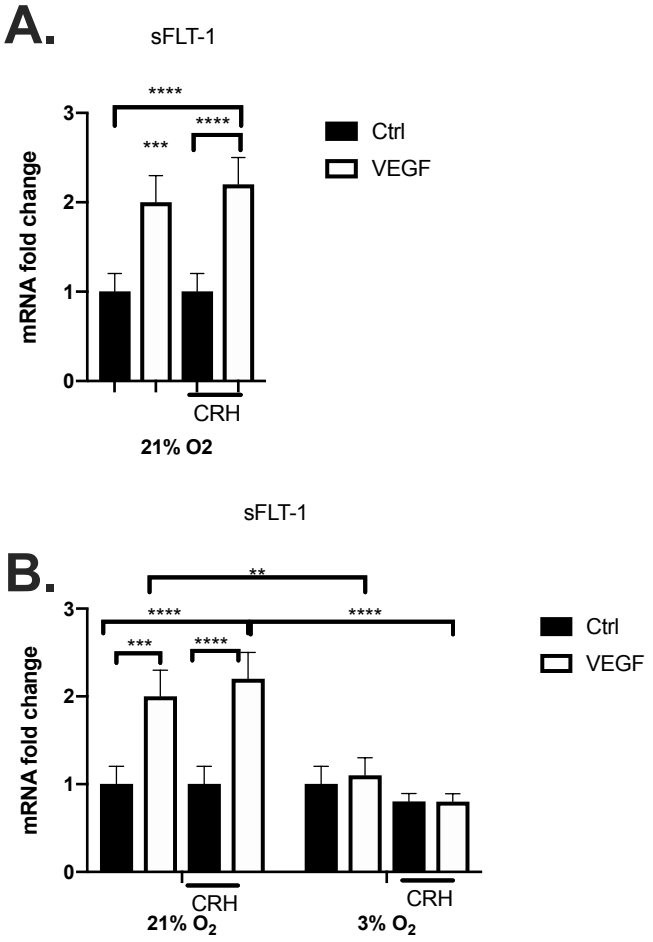


Figure 203

*Hypoxia abolishes VEGF mediated upregulation of sFLT-1 mRNA in HMEC-1 cells. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. HMEC-1 cells were treated with 100nM CRH and 20ng/ml VEGF for 24h. QRT-PCR analysis showed (A) VEGF upregulates mRNA expression of sFLT-1 in HMEC-1 cells at 24h at 21% O2. (B) The effect is lost in 3%O2. CRH has no effect on sFLT1 mRNA under high and low oxygen tension. p= * 0.033; ** .002; *** .0002; ****< .0001; values are mean +/S.D; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

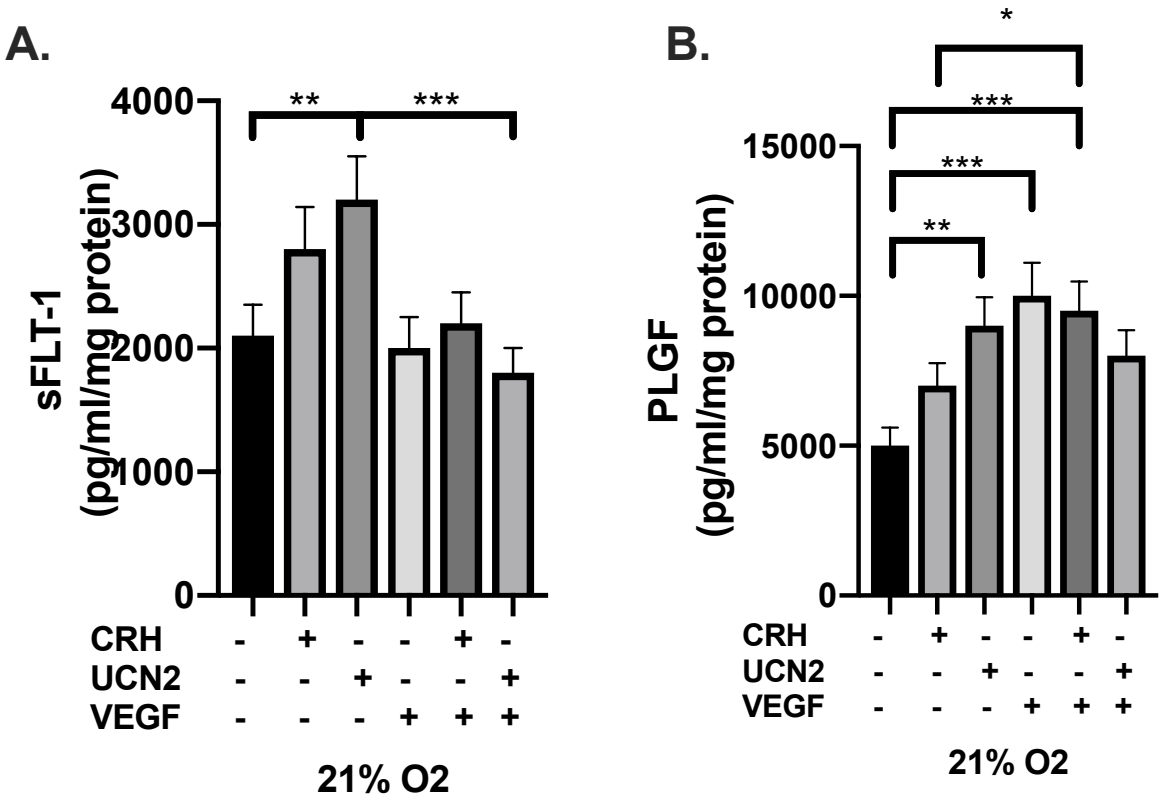


Figure 204

*UCN2 increases sFLT-1 and PLGF protein release at 21%O2 in HMEC-1 cells. VEGF increases PLGF protein release. Experiment protocol; where after 80% confluency, fresh media was added and HMEC-1 cells were treated with 100nM CRH and 20ng/ml VEGF for 24h. Experiment protocol, where after 80% confluency, fresh media was added and HMEC-1 cells were treated with 100nM CRH and 20ng/ml VEGF at 21% O2. Supernatants were collected at 24h at 21% O2 and MSD ELISA assay was done on sFLT-1 and PLGF proteins. . p= * 0.033; ** .002; *** .0002; ****< .0001; values are mean +/S.D; n=3. Data expressed as pg/ml normalised against mg protein. Data were analysed using one – way ANOVA with Tukeys multiple comparison test.*

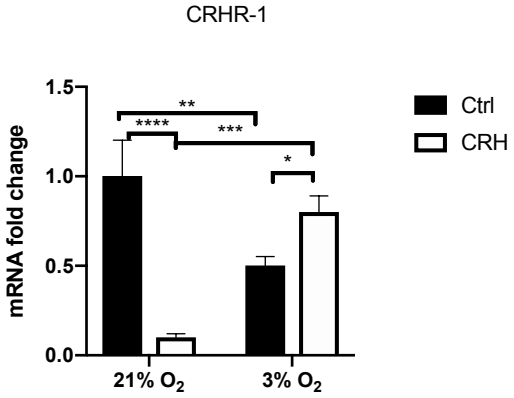


Figure 205

CRH downregulates CRHR1 mRNA expression at 21%O2 in HMEC-1 cells. Hypoxia reverses the effect of CRH on CRHR1 mRNA. Hypoxia downregulates CRHR1mRNA expression. Experiment protocol; where after 80% confluency, fresh media was

*added and some cells were incubated at 3% O2 for 24h. HMEC-1 cells were treated with 100nM CRH and 20ng/ml VEGF for 24h. QRT-PCR analysis showed under high oxygen tension, at 24h, CRH downregulates CRHR1 mRNA expression. Hypoxia reverses this effect. Hypoxia downregulates CRHR1 mRNA. p= * 0.033; ** .002; *** .0002; ****< .0001; values are mean +/S.D; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

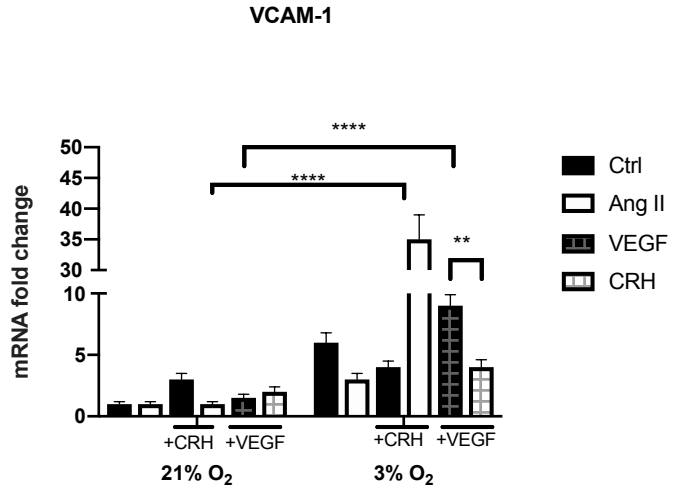


Figure 206

*Hypoxia increases VCAM-1 mRNA but not ICAM-1 mRNA. Experiment protocol; where after 80% confluency, fresh media was added and some cells were incubated at 3% O2 for 24h. HMEC-1 cells were treated with 100nM CRH and 20ng/ml VEGF for 24h. QRT-PCR analysis showed under low oxygen tension, at 24h, VCAM-1 mRNA expression is upregulated. p= * 0.033; ** .002; *** .0002; ****< .0001; values are mean +/S.D; n=3. Data expressed as fold change of gene expression, relative to control 24h, after normalising against B-actin. Data were analysed using two – way ANOVA with Tukeys multiple comparison test.*

Table 79

	21% O2						3% O2					
	Ctrl	Ang II	CRH	CRH + Ang II	VEGF	CRH + VEGF	Ctrl	Ang II	CRH	CRH + Ang II	VEGF	CRH + VEGF
VCAM-1	NS	NS	NS	NS	NS	NS	↑	NS	NS	↑	NS	↓
ICAM-1 (Data not shown)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

HMEC 24H

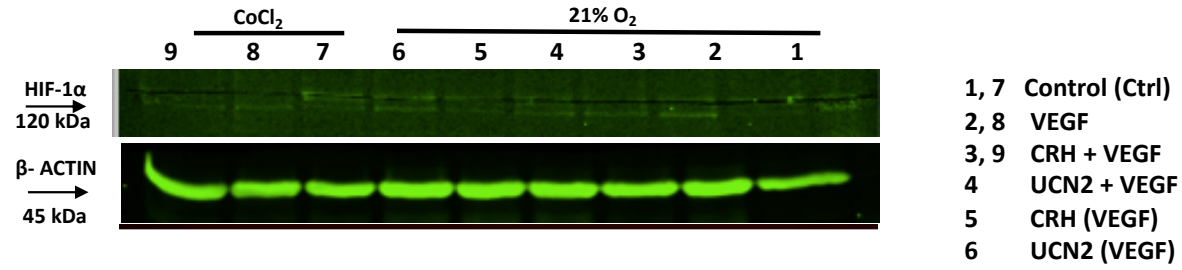


Figure 207

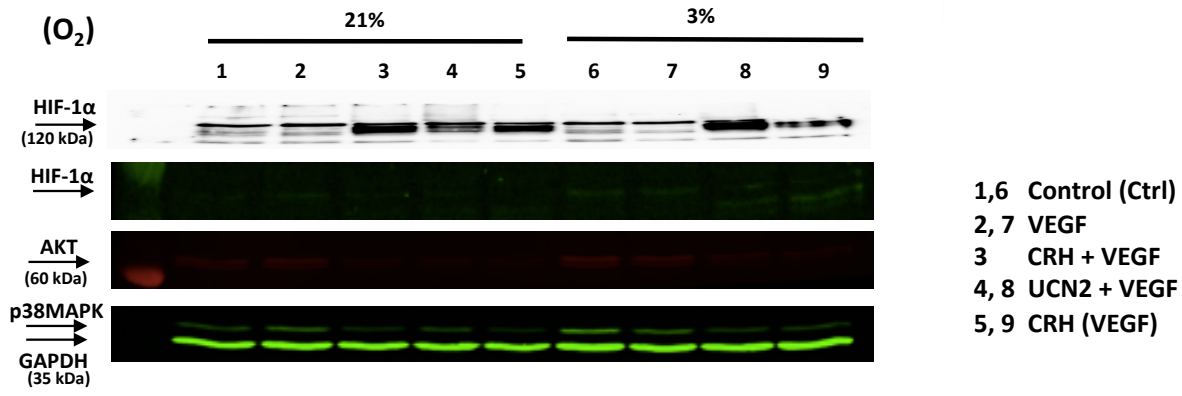


Figure 208

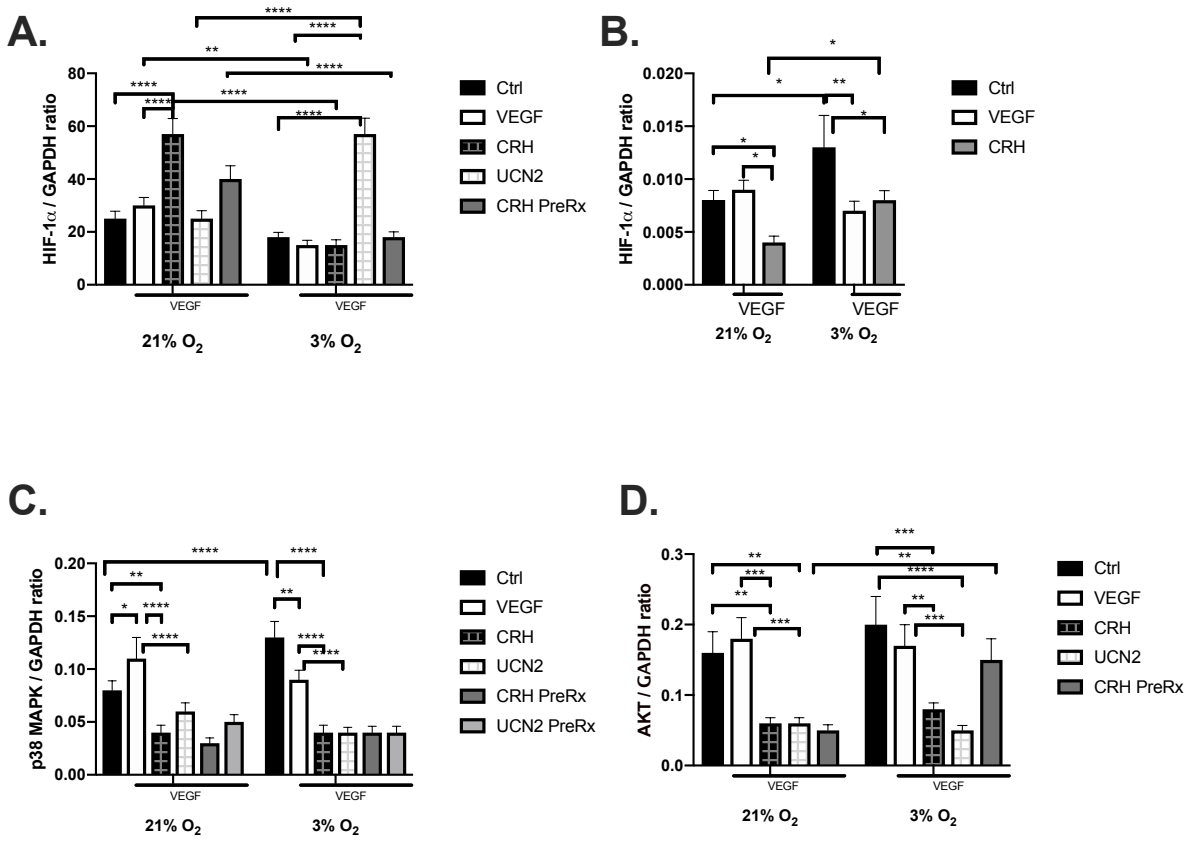


Figure 209

Table 80

24H	21 % O2									3 % O2								
	Ctrl	VEGF	CRH	CRH +VEGF	Ctrl (VEGF)	CRH (VEGF)	UCN2 (VEGF)	UCN2	UCN2 +VEGF	Ctrl	VEGF	CRH	CRH +VEGF	Ctrl (VEGF)	CRH (VEGF)	UCN2 (VEGF)	UCN2	UCN2 +VEGF
HIF-1a/GAPDH ratio	NS	NS	NS	↑	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	↑	NS
HIF-1a/GAPDH ratio (licor)	NS	NS	NS	↓	NS	NS	NS	NS	NS	↑	↓	NS	↓	NS	NS	NS	NS	NS
P38 MAPK /GAPDH ratio	NS	↑	↓	↓	NS	↓	↓	↓	↓	↑	↓	NS	↓	NS	↑	↑	NS	↓
AKT/GAPDH ratio	NS	NS	NS	↓	NS	↓	NS	NS	↓	NS	NS	NS	↓	NS	NS	NS	NS	↓

Figure 210

Western blot (LICOR) analysis showed hypoxia increases HIF-1a expression and VEGF reduces HIF-1a protein expression at 24h. Under high oxygen tension, VEGF increases P38 MAPK expression. CRH and UCN2 reduces p38 MAPK expression. CRH and UCN2 reduces VEGF mediated increase in p38 MAPK expression. Priming cells with CRH and UCN2 for 6h reduces VEGF mediated increase in p38 MAPK expression. Hypoxia increases p38 MAPK expression. Under low oxygen tension, VEGF reduces p38 MAPK expression. CRH and UCN2 further reduces VEGF mediated regulation of p38 MAPK expression. Priming cells with CRH and UCN2 for 6h reduces VEGF mediated regulation of p38 MAPK expression. GAPDH was used as

*a loading control in each sample. Densitometric analysis was performed using Odyssey software and the relative value of the protein expression was normalised against GAPDH. p= * 0.033; ** .002; *** .0002; ****p< .0001; n=3. Data were analysed using a two way ANOVA with Tukeys multiple comparison test.*

7 General Discussion

Taken these results together, it can be concluded that in accordance to previous published results, low oxygen tension impairs fusogenic machinery and biochemical differentiation of trophoblasts. Placenta has an elevated metabolic rate and consumes a significant proportion of the nutrients such as glucose and oxygen that is delivered to the uterus and its contents. However, while there have been many studies on the effects of hypoxemia on fetal cardiovascular, metabolic and endocrine functions, there are limited data on the effects of reduced oxygenation on placental endocrine activities.

Syncytin proteins have a role in placentation, fertilization, and reproductive organ tumors, all of which are closely connected to human reproduction. Syncytin genes are vital for early development. This is supported with the occurrence of embryonic lethality in syncytin A −/− knockout mice. Also, altered syncytin proteins expression have been observed in placental pathologies such as PE, IUGR, GDM(Lee, Keith et al. 2001, Kudo, Boyd et al. 2003, Holder, Tower et al. 2012, Soygur, Sati et al. 2016) and different cancers (Larsson, Bjerregaard et al. 2007, Maliniemi, Vincendeau et al. 2013, Mo, Ouyang et al. 2013).(Chiang, Liang et al. 2009) showed reduced levels of syncytin 1, GCM1, and placental growth factor, which are all essential for vasculogenesis and syncytiotrophoblast formation in PE placentas. Syncytins play a role in immunosuppression events (Mangeney et al. 2007) and cell apoptosis(Knerr, Schnare et al. 2007, Mangeney, Renard et al. 2007, Knerr, Soder et al. 2008, Huang, Chen et al. 2014) in human placenta. Studies show a relationship between syncytin 1 and apoptosis in placentas obtained from preeclampsia (Ishihara, Matsuo et al. 2002, Huang, Chen et al. 2014). Knockdown of Syncytin 1 in BEWO cells results in enhanced apoptosis in BEWO. Thus, alteration in cell cycle progression and apoptosis resulting due to altered syncytin expression may cause abnormalities in PE placentas. Syncytins and their receptors has a key role in the trophoblast fusion [P˙otgens et al., 2004] as well as in fusion of primary cells and cell lines. Several groups have shown that BEWO cells express Syncytin-1 and -2 and that forskolin treatment upregulates their expression (Kudo, Boyd et al. 2003, Vargas, Toufaily et al. 2011). Similarly, my data confirmed an upregulation of Syncytin-1 and -2 on mRNA level after

forskolin treatment. Syncytin proteins need to be further investigated to rule out post transcriptional modifications.

B hCG is the hormone used as a marker for biochemical differentiation of trophoblast cells in studies involving primary trophoblast cells or trophoblast cell line systems(Leisser, Saleh et al. 2006, Butler, Elustondo et al. 2009). Different to beta-hCG, the alpha subunit of human chorionic gonadotropin (alpha-hCG) has been thought to be upregulated already prior to syncytial fusion. Cytotrophoblast and syncytiotrophoblast cells synthesize excess of B hCG and hyperglycosylated hCG. hCG and hyperglycosylated hCG synthesized facilitated myometrial muscle relaxation/ uterine quiescence, trophoblast differentiation, progesterone production, promote invasion as part of implantation, and promote uterine spiral artery angiogenesis and hemochorial placentation, uterine growth, fetal growth, and immune and macrophage suppression(Shi, Lei et al. 1993, Cronier, Bastide et al. 1994, Kraiem, Sadeh et al. 1994, Kurtzman, Wilson et al. 2001). hCG has an extremely long circulating half-life of 36 h. hCG plays a key role in maintaining HSD11B2 expression in placenta, the latter is mediated through activation of the cAMP pathway (Sun, Yang et al. 1998). Forskolin induced cell fusion, via elevation of cAMP, protein kinase A activation and of the transcription factor GCM1, finally activating syncytin-1. This results in BEWO cell fusion and the subsequent protein expression of PP13 and beta-hCG(Wice, Menton et al. 1990, Knerr, Schubert et al. 2005) .
In my experiments, incubation of BEWO cells with forskolin increased the hCG secretion by 8-fold. Likewise, studies showed that cAMP stimulation leads to increased hCG production in BEWOs. Primary trophoblast cells are capable of producing hCG without CAMP stimulation with forskolin or other cAMP-stimulating agents.(Chou 1978, Delidaki, Gu et al. 2011). Similar to the effect observed in trophoblast cell lines(Ringler, Lee-Chuan et al. 1989)observed that forskolin stimulation of primary trophoblast cells resulted in the hCG production. Thus, BEWO cells seem to respond similar to primary cells after being treated with forskolin.

Interestingly, (Al-Nasiry, Spitz et al. 2006) and (Orendi, Gauster et al. 2010)] observed that the fusion process is not coupled with the biochemical differentiation of trophoblast cells. (Al-Nasiry, Spitz et al. 2006) showed that stimulation of non-fusing trophoblast cell line JEG-3 with forskolin results in increased hCG production without occurrence of fusion. (Orendi,

Gauster et al. 2010)observed that after blocking fusion, forskolin-stimulated BEWO cells observed enhanced hCG production.

Similarly, to these findings, my data showed that transcription of syncytin and hCG release are barely detectable after 24 h of forskolin treatment whereas hCG production was already increased by 8-fold suggesting that biochemical differentiation is progressing more rapidly in BEWO cells (data not shown). Another possibility is that hCG production might accelerate the BEWO cell fusion process in an autocrine manner. In support of this, (Dhar, Karmakar et al. 2004)showed that blocking the endogenous hCG with an antibody resulted in impairment of primary cytotrophoblast fusion. The hCG receptor expression in placental tissue was observed by (Reshef, Lei et al. 1990) and (Shi, Lei et al. 1993) also showed that hCG regulates differentiation of cytotrophoblast in primary cells. In common with other studies of BEWO cells, HCG release was reduced by hypoxia(Kudo, Boyd et al. 2003) (Hu, Killion et al. 2007). It has been shown that exogenous dibutyryl cAMP increases trophoblast fusion and differentiation , syncytin1, hCG release by normoxic trophoblast, which in turn feedback and augment signaling pathway associated with fusion (Esterman, Finlay et al. 1996). It also has been demonstrated that hypoxia inhibits trophoblast cell fusion and differentiation. In hypoxia, despite of an increase in syncytin expression and decrease in hCG release, cAMP pathway is less effective (Alsat, Wyplosz et al. 1996, Nelson, Johnson et al. 1999, Jiang, Kamat et al. 2000). The actual mechanism of differentiation that induced hCG release is impaired. My data was in agreement with these published studies. Since altered differentiation induced by low oxygen tension is associated with disrupted balance between fusion and apoptosis (some of my data showed increased cell debris), proapoptotic cascades need to be further investigated.

The hormones progesterone (P4) and estradiol (E2) have multiple key functions in placentation and fetal growth and development and therefore, they would be important marker molecules for endocrine functionality. Progesterone release increased after forskolin treatment in BEWO cells. This finding is in agreement with the result from (Nulsen, Silavin et al. 1989)and (Maldonado-Mercado, Espinosa-García et al. 2008). Given that BEWO cells are shown to express different progesterone receptors , Progesterone might act in an autocrine manner on BEWO cells(Zachariades, Foster et al. 2011). Also, a cross-talk of hCG might occur

with P4 in the BEWO cell line since (Dhar, Karmakar et al. 2004) showed that blocking of hCG by using an hCG antibody reduced P4 production in primary trophoblast cells. So it might be possible that hCG is an autocrine inducer of P4 production and this might explain a similar pattern of secretion of both hormones after forskolin treatment. Furthermore, progesterone then might have a positive effect on cell fusion as (Noorali, Rotar et al. 2009) observed Syncytin-1 upregulation by progesterone in a trophoblast cell line(Fadhillah, Yoshioka et al. 2014).

My data is in agreement with the study which showed that exogenous dibutyryl cAMP increased production of hCG and progesterone by normoxic trophoblast but not by hypoxic cells(Esterman, Finlay et al. 1996). The possible mechanism has been speculated that oxygen tension may either affect reactive oxygen species or GCMa, a placenta-specific transcription factor, which recently has been reported to regulate syncytin expression and consequently cell fusion in trophoblasts (Matouskova, Blazkova et al. 2006).

Under hypoxic conditions, the syncytialised BEWO cells are unable to secrete progesterone may reflect an inhibitory effect of severe decrease in maternal and/or fetal oxygenation on placental progesterone production. The increase seen in progesterone levels under hypoxic conditions in undifferentiated cells may be similar to data in published reports which showed a trend towards an increase in utero-placental progesterone production with hypoxia. This may be due to the elevation in placental PGE_2 production that occurs with hypoxia, since PGE_2 has been reported to increase production of placental progesterone in sheep in vitro.

The enzyme cytochrome P450 17A1 (= steroid 17-alpha-hydroxylase), which is encoded by the CYP17A1 gene, is deficient in the placenta and consequently the placenta cannot produce estrogen de novo. In vivo, the placenta is treated with DHEA and DHEAS as precursor molecules for estrogens and the enzymes sulfatase, 3β-HSD, 17β-HSD, and P450 aromatase are involved in the hormones production such as estrone and estradiol (E2). Similar to the in vivo situation, it is required to supply BEWO cells with precursor molecules for E2 production. Ugele and Simon [1999] showed that BEWO cells can uptake DHEA-S. Also Pattillo et al. [1972] observed that BEWO cells can synthesize E2 from DHEA. Furthermore, Bahn et al. [1981] saw that BEWO cells can produce E2 when FCS is available in the cell culture. A previous study also

reported that fetal bovine serum contains the precursor molecules, which promotes production of estradiol in FBS concentration-dependent manner in BEWO cells.

(Jeschke, Mlcak et al. 2007) observed a small rise in production of cortisol after treating BEWO cells with glycodelin A N-glycan. A study reported that a reduction in fetal blood O_2 saturation and content, was associated with a rise in lactate and Cortisol level. Elevated cortisol concentration in preeclampsia placenta, associated with prematurity and low birth weight (Aufdenblatten, Baumann et al. 2009), has been reported to inhibit proliferation of trophoblasts via glucocorticoid receptor (Hsu, Lan et al. 2009). My result showed hypoxia increases cortisol in undifferentiated BEWO cells and cortisol release remains same in differentiated BEWO cells under both oxygen tensions.

Surprisingly, in undifferentiated cells, the expression of syncytin genes, P4, E2 and cortisol were significantly upregulated in hypoxic condition compared to normoxia. This suggests that the reduced upregulation of genes and hormones in 3% compared to 20% may be the result of secondary events triggered by hypoxia during differentiation, which in turn impair fusogenic machinery and endocrine capacity of BEWO. Therefore, in the absence of differentiation stimuli, hypoxia may be able to directly induce syncytins, P4, E2, and cortisol, while in the presence of differentiation stimuli it may indirectly cause an impaired upregulation, resulting in incomplete differentiation of the cells. The other possibility is that hypoxia is affecting and thus increasing basal levels of syncytin genes, thereby potentially blunting any observed effect of cAMP activator. Therefore, hypoxia may not, in real, impair the differentiation machinery. It would be further interesting to analyze protein content of syncytin genes, and thus rule out post transcriptional modifications. Although the most representative system of in vivo situation in the placenta available are primary trophoblast cells or placental explants, they are characterized by poor reproducibility. Similar issues might be present when performing experiments with primary trophoblast cells, however this might be less present as primary cells consist of one homogenous cell type.

The mechanism of formation of anchoring villi has been illustrated by several groups using placental explants cultured ex vivo under varying oxygen concentrations. First trimester villi, explanted onto a 3D extracellular matrix under high oxygen tension (20%), form new sites

spontaneously at their tips(Vicovac and Aplin 1996, Genbacev, Zhou et al. 1997, Aplin, Haigh et al. 1999), whereas explants cultured in 2 or 3% oxygen show increased production of EVT(Genbacev, Zhou et al. 1997, Gerber, Condorelli et al. 1997) (Caniggia, Mostachfi et al. 2000). Proliferation of Cytotrophoblast has long been known to be excessive in hypoxia (Fox 1964, Kingdom and Kaufmann 1997), although the oxygen sensor in placenta still remains to be identified. Moreover, as predicted from observations in vivo, HIF-1α mRNA expression are several-fold higher after culture in low oxygen.

Now, following up on their earlier finding that TGF-β3 mRNA levels decreases after the transition of oxygen(Esterman, Finlay et al. 1996), the Caniggia group showed that levels of TGF-β3 decreases when HIF-1α mRNA is decreased by antisense treatment. Because decrease in TGF-β3 has no effect on the levels of HIF-1α(Caniggia, Mostachfi et al. 2000) , it was concluded that TGF-β3 lies downstream of HIF-1α. Beyond its effects on TGF-β3, HIF-1α inhibits the expression of molecules that are required for migration such as integrin α1β1 and MMP9(Genbacev, Joslin et al. 1996, Caniggia, Mostachfi et al. 2000). It was further argued that TGF-β3 acts in an autocrine fashion to modulate these events. Certainly, TGF-β inhibits migration of trophoblast in culture (Irving and Lala 1995), but this interpretation is not straightforward. This is because First, proliferation, migration of cytotrophoblast and decidua colonization occur in vivo both before and after the transition of oxygen(Irving and Lala 1995). Second, column formation de novo occurs in cultures in 20% oxygen (Genbacev, Zhou et al. 1997, Aplin, Haigh et al. 1999). Third, TGF-β is produced excessively in decidua, through which migration of trophoblast occurs(Graham, Lysiak et al. 1992). Thus, reports both in vivo and in vitro suggest that the placenta adapts to transition of oxygen environment by maintaining its essential functions — hormone production, and nutrient transfer, anchorage, EVT migration, — but that it regulates the rate of each process at specific developmental stages to meet requirements of fetus. Overall, my data is in agreement with studies which showed secretion of hCG, progesterone were decreased in hypoxia when compared to trophoblast in normoxia(Esterman, Finlay et al. 1996). Conservation of oxygen and ATP by reducing hormone synthesis may contribute to survival of trophoblast in hypoxia.

In BEWO syncytialised cells, CRH potentiated forskolin actions on syncytin 1 and 2 genes expression. Since placental CRH expression in trophoblast cells begins when the cells differentiate toward syncytialization, the promotion of syncytialization by CRH could explain the exponential increase of CRH detected in maternal plasma as pregnancy progresses towards term, particularly as syncytial nuclear numbers increase exponentially across gestation (Grammatopoulos 2008, Fogarty, Mayhew et al. 2011). This may provide the mechanism that links maturation of the placental structure to the timing of birth.

Low oxygen tension blocks CRH mediated potentiation of syncytin1 gene expression in undifferentiated and differentiated cells. Hypoxia abolishes CRH mediated potentiation of syncytin 2 gene expression in differentiated cells. This could be possibly because altered oxygen tension is affecting at receptor level or post receptor level. In contrast, CRH did not affect BHCG, P4, E2, cortisol release in undifferentiated and differentiated cells, under both high and low oxygen tensions. While B HCG release increases during early pregnancy, CRH increases exponentially around second trimester of pregnancy. Although this is in contrast to previously published literature which showed HCG secretion to be stimulated by CRH, through paracrine or autocrine mechanisms, or both, because it is also produced by both the cytotrophoblast and the syncytiotrophoblast(Berga, Nitsche et al. 2016). This suggests CRH might play a role in accelerating morphological differentiation in trophoblast. CRH cannot reverse or prevent the reduction of syncytin gene expression and B HCG, P4 release induced by low oxygen tension and is thus unable to restore the syncytialization. It would be worthwhile to further investigate if the effects are reversed on exposing the cells from low to high oxygen tension.

BEWO cells are invitro model of syncytiotrophoblast, fuses following syncytialization with forskolin to produce a large multinucleated cellular monolayer. Effective syncytialization was determined by quantifying syncytin 1 and 2 genes expression as well as hCG output, post treatment with forskolin. Endocrine capacity of cells was measured by measuring E2, P4 and cortisol. In accordance to previously published data, following syncytialization, both syncytin 1 and 2 genes are upregulated and B HCG, E2, P4, cortisol is significantly released. Furthermore, under low oxygen tension, syncytialization is affected where syncytin genes and release of B HCG, E2, P4, cortisol is reduced. CRH potentiates the actions of forskolin on syncytin 1 gene expression, but has no effect on syncytin 2 and hormones under high oxygen

tension. CRH cannot reverse or prevent the reduction of syncytin gene expression and B HCG, P4 release induced by low oxygen tension and is thus unable to restore the syncytialization.

Previous studies have located both types of CRH-R in human placenta. (Florio, Franchini et al. 2000) CRH-R staining appears almost exclusively to be membrane-distributed, mainly in syncytiotrophoblast and some around vessels. Excess maternal plasma CRH release is associated with downregulation of placental CRHRs in pre-eclamptic women(Grammatopoulos 2008). It is not clear if excess CRH released causes desensitization of receptors or is it being released to upregulate receptors so as to restore to normal level. Given that CRH is unable to restore the trophoblasts fusogenic machinery, the CRHR 1 and 2 expressions were investigated in undifferentiated and differentiated cells under high and low oxygen tension. Furthermore, the effect of CRH on the receptors were investigated. CRHR1 is upregulated during differentiation under high oxygen tension (CRHR1 is more likely to be expressed at cytotrophoblasts level. In case mRNA is translated into protein, this suggests that differentiated BEWO cells are more responsive to CRH cellular action) and is downregulated under low oxygen tension, thus indicating that CRHR1 is most likely involved in making cells responsive to CRH cellular actions, thus facilitating differentiation. It is unclear as why CRH has no effect on the transcription of both CRHR1 and CRHR2 under high oxygen tension, although the protein levels and functional aspects of cell has not been assessed yet. Forskolin, on the other hand, changes gene expression of the CRH receptors. The CRH receptors are thus likely to be regulated by cAMP/CREB cascade. CRH however does not couple to cAMP/CREB cascade, thus has no effect on receptor expression.

However, CRH blocks upregulated CRHR1 during differentiation and brings it to basal levels. This might explain why CRH has not much role to play during differentiation or perhaps only increases syncytin 1 gene. During hypoxia, both receptors are upregulated, CRHR2 more predominately being expressed than CRHR1. Perhaps, there is a fine balance that exists between expression of CRHR1 and CRHR2 in cytotrophoblasts gets disrupted under low oxygen tension. The balance being erred more towards CRHR2 being expressed at higher level. Furthermore, CRH blocks CRHR1 in undifferentiated BEWO cells under low oxygen tension, thus indirectly causing CRHR2 to be expressed at high amounts. Under high oxygen tension, CRH perhaps has no role to play at cytotrophoblast level and hence it is unable to

upregulate its receptors (only a trend towards an upregulation is seen). Perhaps, the excess CRH being released in preeclampsia leads to downregulation of CRHR1 at cytotrophoblast level. Or it is being released to restore the balance between both receptors, but is unable to overcome the effects of hypoxia. A trend towards upregulation of CRHR2 expression is seen, though fails to reach statistical significance. After or during fusion, under high oxygen tension, CRHR1 is expressed in excess amount at syncytiotrophoblasts. Thus causing CRHR1 to be present at higher level than CRHR2. Differentiating cells when exposed to low oxygen tension undergoes downregulation of CRHR1 expression; thus indirectly causing CRHR2 to be expressed at higher amounts. Also, contributing towards the fact that syncytialised BEWO cells are unable to respond to CRH. This in turn contributes towards impairment towards differentiation. This probably causes placental CRH to be released in excess in an attempt to upregulate the protective CRHR1 under hypoxic conditions so as to restore or repair impaired differentiation machinery of trophoblasts. CRH when added to differentiating cells under low oxygen tension has no effect on its receptors. CRH perhaps is hence unable to restore the impairment of differentiation induced by low oxygen tension, because the syncytiotrophoblasts are less responsive to CRH cellular actions. And again, CRH might be being released in excess so as to restore the balance between CRHR1 and 2 in syncytialised BEWO cells. Further experiments are required with antagonists to confirm the above hypothesis.

Given that CRH was unable to restore differentiation machinery, it was further assessed whether activating the CRH receptors by priming cells with CRH would be able to protect trophoblast differentiation from hypoxic effects. Perhaps, it was further considered that mere activation of receptors may not be sufficient to exert protective effects of fusogenic machinery. Hence, a new dimension was added to the experiment. After priming with CRH, extended treatment of CRH for 24h was added along with forskolin to BEWO cells.

Under high oxygen tension, CRHR1 is upregulated during differentiation. By priming cells with CRH, there is further enhancement of CRHR1 expression. The effect of priming cells on CRHR1 activation though by itself is not statistically significant. Adding CRH during differentiation had blocked CRHR1 expression by 50%. However, due to the priming effect, (extended presence of) CRH is unable to reduce CRHR1. Rather, priming makes CRHR1 to remain upregulated

during differentiation of trophoblasts. CRHR2 expression remains unaffected during differentiation and CRH itself has no effect on CRHR2 in undifferentiated and differentiated cells. However, priming syncytialised cells with CRH as well as further extended treatment with CRH enhances CRHR2 expression significantly. this is in alignment with a study(Chen, Allars et al. 2013). Overall, priming cells with CRH (or activation of CRHR) ensures CRHR1 and 2 expression remains upregulated during differentiation.

Under low oxygen tension, we had already observed that CRHR1mRNA is downregulated during differentiation and adding CRH made no difference. CRHR2 remained unaffected during differentiation and adding CRH again made no difference. Priming cells with CRH showed a trend towards upregulation of protective CRHR1, but fails to reach its statistical significance. Again, further presence of CRH makes no difference. During normal pregnancy, perhaps, CRH being increased exponentially during 2nd trimester is in parallel with formation of syncytiotrophoblasts, which is more being formed in first and second trimester. Excess CRH being released during preeclampsia is associated with impaired trophoblast differentiation. It may be possibly being a reason for CRH to be released in excess so as to ensure to prime cells in order to activate CRHR and thus further lead to upregulation of CRHRs during differentiation. However, hypoxia more likely interferes with ability of CRH to activate CRHR during differentiation, further leading to CRHR being less responsive to CRH cellular actions. Thus providing a new insight into role of CRH during trophoblast differentiation.

Forskolin increases syncytin 1 and 2 genes expression and CRH further potentiates it under high oxygen tension. However, priming cells with CRH prevents the ability of extended CRH treatment to potentiate fusogenic machinery. Low oxygen tension interferes with upregulation of syncytin genes and the ability of CRH to potentiate the expression of these genes. Priming cells with CRH, with or without extended CRH treatment has no impact on fusogenic machinery. Also, priming cells with CRH alone or along with extended CRH treatment does not make any significant changes in biochemical differentiation, as well as endocrine capacity of BEWO cells under both oxygen tension.

In the above experimental approach, both priming cells with CRH, followed by incubation of BEWO cells with CRH and forskolin was done under high and low oxygen tension. Another experimental approach was tested where the stage of priming cells with CRH were done

under high oxygen tension, following which change in oxygen tension during differentiation stage was introduced (data not shown). That is, after priming under high oxygen tension, cells with forskolin and CRH were incubated under high or low oxygen tension. Thus, testing whether hypoxia was affecting and thus interfering with activation of CRHR, and that, introducing low oxygen tension after priming cells may allow CRH to restore trophoblast differentiation. Nevertheless, this approach gave the same results as shown in (figure).

Normal placental protein synthesis requires approximately 30% of human placental oxygen consumption (2). Thus, as in cases of IUGR and preeclampsia, with reduced supply of oxygen and nutrients, the placenta conserves energy by shutting down its protein synthetic machinery thereby declining its rate of cell proliferation, which are essential for cells to respond to and survive hypoxia (Palmer, Moore et al. 1999, Carter 2000, Keyes, Armaza et al. 2003, Grocott, Martin et al. 2009). This regulation of protein synthesis occurs at the level of translation primarily and the central regulator of this translation is the mammalian target of rapamycin (MTOR) pathway(Wullschleger, Loewith et al. 2006, Roos, Jansson et al. 2007). A few studies have shown that presence of MTOR in the placenta is crucial in early development, because MTOR gene disruption is lethal post implantation, consequential of impaired cell proliferation in both the embryonic and extra-embryonic compartments. Additionally, placental MTOR also plays key roles in the regulation of nutrient transporter in placenta and fetal growth(Gangloff, Mueller et al. 2004) (Jansson, Pettersson et al. 2006, Roos, Jansson et al. 2007). MTOR was highly expressed in the syncytiotrophoblast, which is the transporting epithelium of the placenta, this cellular localization is compatible with a nutrient sensing role of the MTOR signaling system. This is because the apical surface of the syncytiotrophoblast is exposed to maternal circulation directly (facilitating 'sensing' of the supply line of the mother) and possible targets for MTOR, such as nutrient transporters, are also present in the cell. Likewise, CRH-R appears almost exclusively to be membrane-distributed, mainly in syncytiotrophoblast and some around vessels. MTOR is expressed at the mRNA level in placenta (Kim, Sarbassov et al. 2002). studies in immortalized cell lines originating from human trophoblast indicate an essential role for MTOR in the regulation of proliferation of trophoblasts(Wen, Abbasi et al. 2005). The MTOR pathway is thought to be a regulator of invasive trophoblast differentiation, which may be involved in the first stage of term preeclampsia(Pollheimer and Knofler 2005). A study reported a direct correlation

between activation of MTOR protein and trophoblast invasion, indicating a crucial role for this pathway during IUGR in the regulation of EVT invasion (Knuth, Johannsen et al. 2014).

Maternal hypoxia via regulation of MTOR pathway may induce IUGR. The best-characterized downstream targets of MTORC1 are the ribosomal protein S6 kinase 1 (p70S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1). MTORC1 via activation of the protein kinase S6K1 and inhibition of the eIF4E inhibitor 4E-BP1 signals to the translation initiation machinery(Wullschleger, Loewith et al. 2006). The activity in the MTOR signaling pathway is measured by The protein expression of the phosphorylated forms of these downstream effectors.

My data showed that under low oxygen tension, phosphorylation MTOR is increased by 10 fold. Forskolin increases phosphorylation of MTOR by 10 fold, which is further blocked when exposed to low oxygen tension. However, this is happening because hypoxia has increased basal pMTOR. Hypoxia reduces MTOR protein expression by 80 % by 1h, but the reduction is overcome by 24h. Hypoxia increases phosphorylation of S6K70 by 2 fold in BEWO cells. Forskolin further does not affect pS6K in both normoxia and hypoxia. Hypoxia does not affect S6K70 expression at 1h but reduces S6K85 expression by 80%. By 24h, Hypoxia increases S6K70 expression by 4 fold, and S6K85 by 2 fold.

Studies have reported that the MTOR protein is elevated during IUGR, a condition characterized by a failure of the maternal supply line to supply adequate nutrients to the placenta. However, placental phosphorylated S6K1 (Thr-389) expression, a down-stream target of MTOR and a measure of the activity of the MTOR signaling pathway, was markedly down-regulated in IUGR placentas (Roos, Jansson et al. 2007) (Arroyo, Brown et al. 2009). Since fetal growth is determined by supply of nutrients primarily, the reduced placental MTOR activity, increased 4EBP1 activity and reduced trophoblast invasion is thought to be a direct cause of IUGR.

hypoxia plays a dual role of inducing proliferation and integration of trophoblast early in pregnancy, but on the other hand, during late pregnancy, it is associated with placental dysfunction and preeclampsia (Pringle, Kind et al. 2010). HIF expression is associated with many physiological and pathological processes in the placenta(Patel, Landers et al. 2010).

Preliminary evidence showed that HIF-1α was expressed in the cytotrophoblasts of placenta and was reduced as pregnancy progresses [10]. HIF-1α mediate the trophoblast differentiation based on the availability of oxygen (Genbacev, Zhou et al. 1997). Enhanced placental HIF-1α expression were observed in the early gestational stage [11]. furthermore, involvement of HIF-1α has been suggested in the potential pathological mechanisms of several pregnancy complications (Patel, Landers et al. 2010)[. Enhanced HIF-1α stabilization was reported in PE placentas, although one of the studies suggested that upregulation of HIF-1α might be hypoxia independent in PE(Tal 2012).

Studies in which BEWO cells were exposed to severe 1% hypoxia suggest as a result of HIF-1 alpha protein stabilization, MTOR activity is downregulated and MTOR suppressor REDD1 expression is increased. Under hypoxic conditions, REDD1 was known as an essential mediator of the anti-apoptotic effect of HIF-1α. REDD1 is also thought to contribute to apoptosis and DNA damage during cell stress (Land and Tee 2007, Wolff, McKay et al. 2014). Enhanced REDD1 levels in placentas from an early gestational stage, PE, and abnormal MTOR expression was detected in placentas from PE, IUGR(Fahlbusch, Hartner et al. 2015, Weel, Ribeiro et al. 2015). Oxygen availability regulates amino acid transport in placenta through MTOR. This is supported by reports obtained from high altitude pregnancies, which showed decreased 4ebp1 and AKT phosphorylation, downstream targets of MTORc1 and 2. MTOR is a substrate for AKT(Gangloff, Mueller et al. 2004, Murakami, Ichisaka et al. 2004). AKT signaling is thought to play a central role in placental growth regulation. This was confirmed in *AKT1*-null mice, which display IUGR (Yang, Tschopp et al. 2004, Yung, Calabrese et al. 2008). AKT inactivation led to placental hypotrophy and structural abnormalities which is thought to contribute to placental insufficiency and subsequent fetal growth impairment. Previous studies reported that the AKT activation via increases in eNOS activity results in vasorelaxation in endothelial cells (Luo, Fujio et al. 2000, Hisamoto, Ohmichi et al. 2001, Hisamoto, Ohmichi et al. 2001). It was observed that Hypoxia regulates NO production and eNOS activity via AKT activation in porcine coronary artery endothelial cells(Chen, Lawrence et al. 2004). PI3K and AKT play a key role in regulating angiogenesis and tumor growth via upregulation of *VEGF* and *HIF-1* expression.

HIF-1α is a substrate for various kinase pathways, including PI3K –AKT pathways (Minet, Arnould et al. 2000). Only in last 5 days of pregnancy, phosphorylation of AKT is decreased in

placenta obtained from mice exposed to normobaric hypoxia. Although MTOR activity is increased when the insult occurs throughout pregnancy. My data is in alignment with this which shows that hypoxia increases MTOR phosphorylation in BEWO cells. Under low oxygen tension, at 1 hour, AKT expression was downregulated, which is overcome over 24h. Phosphorylation of AKT needs to be further investigated. Though forskolin decreases activation of pAKT and hypoxia blocks this, forskolin has no effect on expression of AKT under normal and high oxygen tension.

So, overall, hypoxia increases basal pMTOR and pS6K70. Forskolin increases pMTOR but not pS6K 70 under normal oxygen tension. In hypoxia, because basal pMTOR is increased, the upregulation of pMTOR seen during differentiation is not evident. Hypoxia has no effect on pS6K70 during differentiation. Hypoxia reduces MTOR protein expression and S6K85 expression, but not S6K70 expression at 1h. By 24h, reduction of MTOR expression is overcome and S6K70 and S6K85 is increased.

Although AMPK expression has been shown to be unaffected by altered oxygen tension and that hypoxia affects activity and expression of MTOR, phosphorylation of AMPK needs to be further examined. This is because it is the phosphorylation of AMPK that has been known to constitute one possible mechanistic link between decreased ATP availability in hypoxic mouse placenta and inhibition of MTOR signaling, ensuring that amino acid transporter activity is aligned with cellular energy status. Conversely, inhibition of AMPK has been proposed to contribute to activation of placental MTOR and amino acid transport activity in maternal over nutrition(Huckle 2017). Furthermore, hypoxia reduces AMPK expression in syncytialised BEWO cells, although their expression levels remains unaffected under high oxygen tension.

HIF-1α is also a substrate for several kinase pathways, including MAP kinases, ERK and p38(Minet, Arnould et al. 2000). MAPK signal pathway is one of the key pathways involved in regulation of the pathogenesis of preterm preeclampsia. MAPK and/or ERK /MAPK signaling is a common pathway downstream to many markers or molecules that are associated with

preeclampsia such sFLT-1, and angiotensin in maintaining endothelial function, HB-EGF in invasion and anti-apoptosis upon hypoxia (Ahmad, Hewett et al. 2011, Herse and LaMarca 2013, Shu, Liu et al. 2014). My data shows that hypoxia inhibits p38 MAPK protein expression in undifferentiated (80%) BEWO cells.

In several cell types, MAPKs are regulated by cAMP. Both p38 MAPK and ERK1/2. activation has been observed to be in association with placental trophoblast differentiation(Daoud, Amyot et al. 2005). Forskolin treatment induced a rapid and potent phosphorylation of ERK1/2 and p38MAPK; this cascade involved PKA–AKAP interactions which further led to downstream phosphorylation of CREB-1/ATF-1 via ERK1/2-dependent but p38MAPK-independent mechanisms. Intriguingly, both ERK1/2 and p38MAPK were associated with forskolin-induced release of hCG, suggesting the additional pathways to be present that are p38MAPK-dependent but CREB-1/ATF-1-independent. Forskolin treatment of BEWO cells upregulated syncytin, OASIS, GCMa, syncytin 2 receptor, AKAP. The effects of Forskolin on all these genes were suppressed by p38MAPK inhibition chemically whereas only specific genes were sensitive to inhibition of ERK1/2. My data shows that hypoxia reduces p38 MAPK protein expression in undifferentiated and syncytialised BEWO cells (80%). Investigation of phosphorylation of p38 MAPK and ERK is further required. This would further provide evidence for reduction of hCG during hypoxia during trophoblast differentiation. Also, it has been observed that hypoxia increases the ERK phosphorylation, followed by *HIF-1α* stabilization and activation, which consequently increases *HIF-1α*-dependent transcriptional activation of *VEGF* in hamster fibroblasts(Berra, Roux et al. 2001) and that PLGF promote the trophoblasts syncytialization (Morrish, Bhardwaj et al. 1987, Garcia-Lloret, Morrish et al. 1994, Crocker, Strachan et al. 2001, Yang, Lei et al. 2003). It has been further observed that under hypoxic conditions in the trophoblast-derived BEWO cell line , angiogenic factors necessary for the development of placenta are activated via AKT - MTOR signaling pathway (Fujita, Tanabe et al. 2010). My data shows PLGF is further upregulated by forskolin in hypoxia, although the release is suppressed. It has been earlier shown that my data is somewhat in alignment with a study which showed induction of hypoxia responsive genes was suppressed by dibutyryl cAMP in pc12 cells. Overall, AKT – MTOR- p38 MAPK pathway may be involved in cAMP induced PLGF production during trophoblast differentiation under altered oxygen tension.

During human placentation, mononuclear cytotrophoblasts fuse to form multinucleated syncytia ensuring hormonal production and nutrient exchanges between the maternal and fetal circulation. Syncytial formation is essential for the maintenance of pregnancy and for fetal growth. Regulation of trophoblast fusogenic machinery and hormonogenesis is achieved by coordinated action of a number of signaling molecules. To investigate the effect of hypoxia on activation and expression of various signaling molecules such as AKT, CREB, HIF, AMPK, MTOR, S6K and p38 MAPK pathways involved in trophoblast differentiation and hCG secretion, BEWO cells were incubated with or without forskolin (cAMP activator) under high and low oxygen tension. The cAMP signaling pathway is the major route to trigger trophoblast fusion and its activation results in phosphorylation of specific intracellular target proteins, in transcription of fusogenic genes and assembly of macromolecular protein complexes constituting the fusogenic machinery at the plasma membrane. Specificity in cAMP signaling is ensured by generation of localized pools of cAMP controlled by cAMP phosphodiesterases (PDEs) and by discrete, spatial and temporal, intracellular protein kinase A (PKA) activation organized by A-kinase-anchoring proteins (AKAPs) and signal termination organized by protein phosphatases (PPs). In both primary human trophoblast cells and choriocarcinoma-derived cell lines the cAMP/PKA cascade, via CREB-1 and ATF-1 phosphorylation, regulates GCMa and syncytin-1 expression and hCG production(Keryer, Alsat et al. 1998, Knofler, Saleh et al. 1999, Knerr, Schubert et al. 2005). Other kinases such as protein kinase C (PKC) might also synergistically interact with cAMP/PKA to stimulate morphological differentiation and hCG production in trophoblasts (Andersen, Milsted et al. 1988). My data shows forskolin increases Phosphorylation of CREB and decreases Phosphorylation of AKT and this is in support of data which showed maximum BEWO cell fusion is a complex process that requires increased intracellular cAMP coupled with decreased intracellular calcium levels and inhibition of PI3K/AKT signaling. In islet cells, CREB has been found to promote cell survival by upregulating insulin receptor substrate 2 (IRS2) gene expression and thereby activating the Ser/Thr kinase AKT. Similar to AKT, studies indicate that cAMP, an intracellular signal that regulates cell proliferation, differentiation, and apoptosis (Boynton 1983, Dumont, Jauniaux et al. 1989, Richards 2001), as well as AKT activity either in a positive or negative manner(Cass, Summers et al. 1999, Filippa, Sable et al. 1999, Forti and Armelin 2000, Gonzalez-Robayna,

Falender et al. 2000, Kim, Jee et al. 2001, Wang, Liu et al. 2001). Despite its mitogenic signal, cAMP inhibits AKT in a manner that requires PKA activity(Cass, Summers et al. 1999, Filippa, Sable et al. 1999, Forti and Armelin 2000, Gonzalez-Robayna, Falender et al. 2000, Kim, Jee et al. 2001, Wang, Liu et al. 2001). AKT inactivation has been shown to cause placental structural abnormalities and hypotrophy that is likely to result in placental insufficiency and subsequent fetal growth impairment. AKT1-null mice display IUGR thus suggesting a central role of AKT signaling in placental growth (Yang, Lei et al. 2003, Yang, Tschopp et al. 2004, Yung, Calabrese et al. 2008). AKT activation via increase in eNOS activity results in vasorelaxation in endothelial cells(Luo, Fujio et al. 2000, Hisamoto, Ohmichi et al. 2001, Hisamoto, Ohmichi et al. 2001) (Northcott *et al*. 2002). in porcine coronary artery endothelial cells, Hypoxia via activation of AKT signaling was observed to regulate eNOS activity and NO production (Chen, Lawrence et al. 2004). My data shows Hypoxia blocks the forskolin mediated reduction of AKT phosphorylation, which further is likely to contribute towards impaired fusion. PI3K and AKT via upregulation of VEGF and HIF-1 expression, is thought to regulate tumor growth and angiogenesis. *HIF-1α* is a substrate for several kinase pathways, including PI3K –AKT pathways(Minet, Arnould et al. 2000). Though forskolin decreases activation of pAKT and hypoxia blocks this, forskolin has no effect on expression of AKT under normal and high oxygen tension. PI3K pathway has been shown to induce the expression of various angiogenic factors such as VEGF, PLGF and its receptor VEGFR1. PI3K induces AKT phosphorylation and activity, which in turn stimulates synthesis of transcription factors such as hypoxia-induced factor-1a (HIF-1 alpha), eventually resulting in enhanced VEGF and VEGFR1 expression (Choi, Park et al. 2011). Thus, hypoxia via PI3K/AKT pathway initiates survival responses such as HIF-1 alpha expression as well as induces apoptosis (Greijer and van der Wall 2004). The PI3K-AKT pathway regulates normal functions of cell such as proliferation, survival, and glucose metabolism. Thus, hypoxia can either activate or inactivate the PI3K-AKT signaling pathway Depending on the cell type. For example, hypoxia activates the pathway in PC12, HeLa cells, HT1080, [20e22], but is inactivates in BEWO, BEWO31, HepG2, and JAR placental cells and in primary cytotrophoblast cells [23e25] under hypoxic conditions. In PE placental cells, hypoxia inactivates the PI3K–AKT pathway. in in vitro models of human placental hypoxia, it was demonstrated that high levels of sFLT-1 at baseline and that inhibition of the PI3K-AKT pathway by a PI3K- specific inhibitor leads to a decreased sFLT-1 level and unchanged or increased VEGF and PLGF levels. My data is in alignment with the above studies and showed

that under low oxygen tension, at 1 hour, AKT expression was downregulated, which is overcome over 24h. Phosphorylation of AKT needs to be further investigated. Reduction of AKT under low oxygen tension may further provide a link towards reduction of angiogenic factors such as PLGF.

Oxygen availability regulates placental amino acid transport through MTOR, as evidence by placenta from pregnant women exposed to hypobaric hypoxia by residing at high altitude, which exhibit reduced phosphorylation of 4ebp1 and AKT, downstream targets of MTORc1 and 2. AKT upregulates MTOR. MTOR, which is a substrate of AKT, is essential for growth and proliferation of early mouse embryonic stem cells(Gangloff, Mueller et al. 2004, Murakami, Ichisaka et al. 2004). MTOR deficient embryos die soon after implantation as a consequence of impaired cell proliferation in both the embryonic and extra-embryonic compartments. These findings suggest that MTOR may play a key role in regulating cell growth and proliferation of trophoblasts(Wen, Abbasi et al. 2005). AKT phosphorylation is reduced in placenta of mice exposed to normobaric hypoxia only in last 5 days of pregnancy, although MTOR activity is increased when the insult occurs throughout pregnancy(Matheson, Veerbeek et al. 2016). My data is in partial alignment with this which shows that hypoxia increases MTOR phosphorylation and decreased AKT expression in BEWO cells.

AMP-activated protein kinase (AMPK) is key in the regulation of cellular energy metabolism (Milan, Jeon et al. 2000). AMPK regulates ATP synthesis and inhibit activation of MTOR (Rehman, Shehzad et al. 2014). Increased MTOR mRNA expression has been shown in placenta. Enhanced expression of MTOR- positive regulators Irs1, Pik3r1, RpS6Ka2, and VEGF-C has been observed while there was a downregulation in the MTOR-negative regulator Prkg2, subunit of AMPK. VEGF-C genes are involved in promoting cell motility and survival and cell growth, implying that, similar to Irs1 and Pik3r1, stimulation could be due to a survival mechanism activated by hypoxic stress(Milan, Jeon et al. 2000) (Roux et al. 2003). My data shows hypoxia increases VEGF mRNA in BEWO. During hypoxia, in syncytialised BEWO cells, there is inhibition of AMPK, negative regulator of MTOR, most likely in an attempt to promote upregulation of MTOR. However, hypoxia impairs activation of MTOR in differentiating BEWO cells. The fact that hypoxia blocks reduction of pAKT by forskolin may be another attempt to overcome impaired activation of MTOR. My data also shows Hypoxia also impairs PLGF

release in differentiating trophoblasts. PLGF is a product of the syncytiotrophoblasts and its gene is 10-fold upregulated during trophoblast differentiation(Depoix, Tee et al. 2011). Hypoxia inactivates VEGF-A, which is a MTOR regulator(Trinh, Tjalma et al. 2009). Thus during hypoxia, the impairment of VEGF, PLGF further is likely to contribute towards impairment of activation of MTOR in differentiating BEWO cells. Further studies are required to establish the relationship between PLGF and MTOR molecules.

cAMP trigger activation of MTOR via the CREB-mediated IRS2-AKT induction. In islet cells, cAMP induces MTOR activity and that MTOR regulates cellular size of beta cell(Jhala, Canettieri et al. 2003, Balcazar Morales and Aguilar de Plata 2012) , it is considered whether cAMP promotes beta cell growth via this pathway. In BEWO, Forskolin has been shown to inhibit MTOR activity and expression, but my data shows forskolin increase in MTOR phosphorylation, other than increase in CREB phosphorylation. My data shows hypoxia impairs phosphorylation of MTOR (10 fold) and CREB (6 fold) in differentiating cells and thus affects proliferation and survival of trophoblasts. *in-vitro* studies have identified distinct actions of MTOR on the biochemical and morphological differentiation of BEWO trophoblasts. Inhibition of MTOR by rapamycin, reduced forskolin-induced hCG release, whereas syncytin-1 and -2 mRNA expression was substantially augmented. Overall, cAMP-CREB-AKT-MTOR pathway appears to be impaired under hypoxic conditions and is most likely to be controlled by HIF-1 alpha.

Forskolin exposure of INS-1 cells stimulated ribosomal protein S6 phosphorylation, a downstream MTORC1 complex target, which includes MTOR, mLST8, PRAS40, and Raptor (Van de Velde, Hogan et al. 2011). My data showed that PS6K is not affected under both oxygen tensions. Thus the activity of MTOR is not affected during differentiation under both high and low oxygen tension. However, hypoxia increases pS6K, S6K expression. S6K 70 expression is not affected at 1h, but increases at 24h. S6K85 expression decreases by 80% at 1h, which further increases upto 2 fold.

cAMP induces CREB and HIF Pathways in pancreatic β Cells. In response to hypoxia and growth factor signaling, HIF stimulates metabolic reprogramming(Van de Velde, Hogan et al. 2011) (Semenza 2010). Hypoxia has been reported to increase stability of HIF1α, whereas growth

factors appear to stimulate the HIF1α translation (Sengupta, Peterson et al. 2010). Increases in cAMP promote the accumulation of HIF1α in beta cells by activating the MTOR pathway and promote islet viability. MTOR-HIF Pathway mediates GLP-1 effects on β Cell viability. HIF has been reported to promote cell growth by inducing glycolysis and by reducing oxidative metabolism of mitochondria(Semenza 2010). Having seen that HIF induced the stress defense genes expression, it was tested whether cAMP activation protects beta cells against oxidative stress or cell death. On inhibition of MTORC1 activity with rapamycin, these effects were reversed. CREB activity disruption, via transgenic expression of dominant negative CREB inhibitors in beta cells, leads to hyperglycemia and diabetes, partly due to reduction in pancreatic islet mass and partly due to consequential reduction in insulin concentrations (Inada, Hamamoto et al. 2004). Cyclic AMP curbs the hypoxia-inducible factors stimulation under hypoxic conditions in PC12 cells. *HIF-1α* is a substrate for various kinase pathways, including PI3K and the MAP kinases, ERK and p38(Daisuke, Akiko et al. 2010) . Under hypoxic conditions in BEWO trophoblast-derived cells, HIF -1 -AKT–MTOR, ERK activation is important for the production of angiogenic factors. On the other hand, hypoxia induces ERK phosphorylation, followed by HIF-1 alpha stabilization and activation, of which enhances HIF-1 alpha-dependent activation of VEGF as seen in fibroblasts of hamster (Berra, Pagès et al. 2000). VEGF, has been reported to induce syncytialization of trophoblasts(Garcia-Lloret, Morrish et al. 1994, Crocker, Strachan et al. 2001, Li, Dakour et al. 2005). My data shows forskolin increases HIF1a, CREB expression and hypoxia reverses this. Hypoxia increases phosphorylation of CREB, MTOR, S6K, as well as expression of CREB, HIF1a, S6K and reduces p38 MAPK expression. PKA, ERK1/2 and p38MAPK(Johnstone, Sibley et al. 2005, Delidaki, Gu et al. 2011, Matsuura, Jigami et al. 2011). Signaling pathways have been reported to play significant role in the initiation of trophoblast differentiation and activation of syncytin 1 leading to cell fusion(Knerr, Schubert et al. 2005). Specific inhibitors for ERK1/2 and p38MAPK led to defective syncytialization in primary trophoblast cultures(Daoud, Amyot et al. 2005). My data shows hypoxia reduces p38 MAPK phosphorylation as well as forskolin induced p38 MAPK and AMPK expression.

HUVEC: placental CRH has been demonstrated to cause vasodilation in the human-fetal placental circulation via activation of the nitric oxide (NO)/cGMP pathway. Reports indicate that while CRH stimulates NO production, NO itself exhibits negative feedback activity on CRH

production in the perfused placenta. This pathway appears to be impaired in PE and may contribute toward dysregulation of the balance controlling vascular resistance. In PE placental explants there was a significant reduction in CRH/CRH-related peptide-induced cGMP response. It is most likely that activation of NO/cGMP pathway is likely to be one of the pathways to be involved by which CRH inhibits sFLT-1 and this pathway may be further dysregulated under altered oxygen tension. This needs to be further explored. P38 MAPK is essential for angiogenesis. CRH induces VEGF release in human mast cells via p38 MAPK. Hence there may be interactions between peptide hormones and angiogenic factors. This recommends further studies exploring correlation and regulation of sFLT-1, PLGF by CRH in models, such as HUVECs, placenta explants. Given that CRH inhibits sFLT-1/PLGF ratio, it is further worth exploring the effect of CRH on arterial blood pressure and induced restricted fetal growth and glomerular endotheliosis in models such as pregnant mice, which are the key hallmark features of placental dysfunction such as preeclampsia.

Along with angiogenic factors, during pregnancy, the placenta also synthesizes CRH and releases it into the maternal compartment at increasing levels to reach concentrations 1,000 to 10, 000 times of that found in the non-pregnant individual. Expression of placental CRH can be detected from the seventh week of pregnancy and increases progressively until term. In the last 5 to 7 weeks of pregnancy, placental expression of CRH increases more than 20-fold (Woods, Grossman et al. 1994). Placental CRH also is secreted into the fetal circulation, albeit to a lesser extent, resulting in elevated CRH levels in the fetus throughout gestation(Petraglia, Florio et al. 1996, Zhao, Hoheisel et al. 1997). A binding protein (BP) for CRH also exists, and for most of pregnancy it is present in excess of CRH in the maternal circulation. As the CRH-BP binds CRH with greater affinity than the CRH receptor, it effectively suppresses CRH activity. Thus, for most of pregnancy the bulk of the placental CRH is sequestered by CRH-BP. However, during the last 4 weeks of pregnancy CRH-BP levels decrease markedly(Florio, Woods et al. 1997). This coincides with the exponential increase in placental CRH production, which could result in a dramatic increase in CRH biologic activity.

Placental CRH may be part of the fetal-placental stress response mechanism. The placenta is comparable to the hypothalamus in its production of CRH in response to stress. In vitro

studies indicate that agents that increase CRH production by the hypothalamus also increase CRH production by cultured placental cells. These agents include neurotransmitters and neuropeptides activated in response to stress such as prostaglandins E2 and F2α (PGE2 and PGF2α), norepinephrine, acetylcholine, vasopressin, angiotensin II, oxytocin (OT), interleukin I, and NPY. In contrast, progesterone and nitric oxide donors inhibit placental CRH expression in vitro. The physiologic implications of this are that the fetus may be able to mount a stress response by means of placental CRH(Linton, PERKins et al. 1993, Erickson, Thorsen et al. 2001). This may be critical in conditions of fetal stress such as preeclampsia, placental vascular insufficiency, and intrauterine infection.

The human placenta also synthesizes urocortin, a 40–amino acid peptide that is a member of the CRH family, has the same biologic effects as CRH, and binds to CRHR1. The role of placental urocortin in human pregnancy is unknown. Two major CRH receptor (CRH-R) subtypes have been identified, CRH-R1 and CRH-R2 in the placenta(PERKins, Eben et al. 1993, PERKins, Wolfe et al. 1995, BERKowitz, Lapinski et al. 1996). Actions of CRH are mediated by specific cell membrane receptors. CRH binds to CRH-R1 with greater affinity than CRH-R2. These receptors and various subtypes within each group demonstrates tissue-specific expression and possibly contribute to differential actions of CRH on different cell types.

It is well known that along with CRH, serum concentrations of sFLT-1 are significantly higher in women with preeclampsia(Dimitrakova, Dimitrakov et al. 2004, Karumanchi and Bdolah 2004, McKeeman, Ardill et al. 2004, Shibata, Rajakumar et al. 2005, Karumanchi and Stillman 2006) this is further associated with downregulation of CRHR and reduced levels of PLGF. Thus, suggesting that the production of CRH and angiogenic factors appear to be related. While it is believed that impaired trophoblast invasion aggravating placental hypoxia leads to angiogenic imbalance, it has not yet been established a relationship between CRH and impaired trophoblast invasion associated with placental dysfunction. Furthermore, it is worth to explore relationship between CRH and placental vascular development, angiogenic homeostasis and endothelial dysfunction. it will be worth investigating the effect of CRH on FLT1, KDR as well as whether sFLT-1, PLGF, VEGF regulates CRH release. Also, since it is likely

to be an adaptation of fetus to the stress response, investigation of these relationships in fetal membranes, amniotic fluid will give further insights about role of CRH.

Toward the end of gestation, continued placental vascular development is terminated, and this process is accomplished in part through the production of antiangiogenic factors. Multiple groups have shown that sFLT-1, an antagonist to the action of VEGF and placental growth factor, is released from cytotrophoblasts in late pregnancy(Kendall, Wang et al. 1996, Clark, Smith et al. 1998, Nagamatsu, Fujii et al. 2004, Li, Gu et al. 2005). The maternal concentrations of sFLT-1 are relatively low during the first trimester, begin to rise during the second trimester, and achieve relatively high levels during the third trimester. Thus it is proposed that increased placental CRH production throughout pregnancy course stimulates production of angiogenic proteins and inhibit anti angiogenic proteins, to support the placental vascular development. This study provides direct evidence that inhibition of endothelial sFLT-1 secretion and mRNA production as well as PLGF release (overall decrease in sFLT-1/PLGF ratio) by CRH and UCN2 under high oxygen tension functions. This is most likely to be mediated via CRHR2 (given that we see reduction by UCN2 and that HUVEC expresses CRHR2 only). it is most likely that CRHR2 expression under hypoxia is altered, perhaps leading to probably inability to keep the ratio low. Thus, it is likely that during normal pregnancy, CRH most likely could promote placental vascular development. Moreover, excess CRH during placental insufficiency syndrome is released in an attempt to restore altered angiogenic protein balance, which is further supported by hypoxia data which shows overcome of inhibition of sFLT-1 mRNA by CRH. However, the biological significance of CRH mediated reduction of sFLT-1/PLGF ratio during pregnancy and placental insufficiency disorders need further investigation. Also, it will be interesting to see if hypoxia overcomes sFLT-1 blocked by CRH or is it because sFLT-1 is already upregulated and hence CRH is unable to act.

Since sFLT-1 is soluble and contains only the ligand-binding domain; as such, it is most likely packaged within the lumen of the vesicle, which transports it from the ER to the Golgi complex. Soluble proteins have short half-life for secretion, around 50 min for proteins such as albumin and alpha1-antitripsin (Lodish, Kong et al. 1983, Nyfeler, Reiterer et al. 2008). Nevertheless, despite being a soluble vesicle luminal protein, sFLT-1 has a relatively long half-life for its release for approximation of 4 hrs. This distinction is most likely explained by the

fact that the reported rate of transport include the time taken necessary for folding and glycosylation in the Golgi, which may be more elaborative since sFLT-1 is a heavily glycosylated protein (Gu, Lewis et al. 2008, Thomas, Andrews et al. 2009).

The Golgi apparatus is a central hub for membrane trafficking in mammalian cells. Golgi complex receives proteins and lipids that are newly synthesized from the endoplasmic reticulum (ER), modifies many of the proteins, and sorts them to various destinations (Glick and Malhotra 1998, Allan and Balch 1999, Pelham and Rothman 2000). sFLT-1 is detected in the extracellular medium and cell lysates After initial synthesis, after 4 hrs, further reaching its peak in media at 8 hrs. Thus sFLT-1 via vesicular trafficking may be transported on its way to secretion into the extracellular medium through the various secretory organelles including ER, Golgi, and post-Golgi compartments. Cargo capture is a mechanism that is used for the transport of newly synthesized proteins selectively from the ER to the Golgi complex(Balch, McCaffery et al. 1994, Schekman and Orci 1996, Barlowe 2003). For its secretion, secretory transport of sFLT-1 through the Golgi complex is prerequisite. The synthesis of sFLT-1 is believed to be secondary to placental hypoxia in clinical preeclampsia(Maynard, Min et al. 2003, Nagamatsu, Fujii et al. 2004). It has been shown that show that the release of sFLT-1 during both high and low oxygen tension requires functional small GTPases Arf1, Arf6, and Rab11 indicating that trafficking itinerary of sFLT-1 remains unchanged during pathological states. Inhibition of their function during both high and low oxygen tensions leading to disruption of membrane transport from the ER to the Golgi complex, is suggested to block the sFLT-1 release which further results in increased sFLT-1 intracellular concentrations.

Since my data observes that inhibition of sFLT-1 mRNA by CRH is less compared to secretion, and this is most likely due to the time difference (24h mRNA and 1h release). CRH furthermore is unable to reduce sFLT-1 secretion at 24h and 48h. The other possibility is that since secretion of sFLT-1 is reduced to maximum extent, it is likely to lead to intracellular sFLT-1 accumulation. a study showed After initial synthesis, sFLT-1 could be detected in the endothelium extracellular medium and cell lysates after 4 hrs, further reaching its peak in media at 8 hrs, which suggests sFLT-1 may be transported via vesicular trafficking through the various secretory organelles such as the ER, Golgi, and post-Golgi compartments on its way to secretion into the extracellular medium. it is thus helpful to measure sFLT-1 mRNA at 4 -

6hours so as to rule out that sFLT-1 is being trapped somewhere inside the cell, after the production of sFLT-1. Furthermore, investigations are required exploring CRH FLT1 expression. This could also be due to the fact that transcription is measured at 24h and sFLT-1 release inhibition is observed at 1h. transcription should be measured by 4-6h. CRH inhibits sFLT-1 and PLGF release but not VEGF release at 1h. Most likely CRH regulates FLT1 receptor and not KDR. CRH inhibits sFLT-1 1 at 1h and PLGF at 1h but overcomes at 24h and 48h.The molecular basis for the difference in regulation of sFLT-1 and PLGF by CRH under altered oxygen tension is thought to involve placental specific transcription factors acting through distinct response elements in CRH promoter region. Since, sFLT-1 inhibits endothelial tube formation by blocking placental growth factor and vascular endothelial growth factor; it is further important to investigate the functional effects of CRH under high and low oxygen tension.

In preeclamptic placenta, there is a downregulation of CRHRs and is associated with excess CRH release and sFLT-1 release. In this study, it is observed that CRH via CRHR2 mRNA upregulation inhibits sFLT-1/PLGF ratio. Hypoxia upregulates CRHR2 and potentiates CRH's actions on CRHR2 upregulation. Hypoxia upregulates sFLT-1 PLGF release via CRHR2 upregulation. The inhibitory effect on CRH on sFLT-1 mRNA is overcome in hypoxia by CRHR2 upregulation.

The observation that CRH inhibits sFLT-1/PLGF ratio via activation of CRHR2 further is in alignment with the argument the CRH family of peptides regulates angiogenesis through VEGF expression levels as seen in various settings. In rat vascular smooth muscle cells (SMCs), activation of CRHR2 by UCN 2 treatment reduces VEGF expression levels(Bale, Giordano et al. 2002, Cao, Papadopoulou et al. 2005). In contrast, CRH increases VEGF secretion in human mast cells and VEGF mRNA levels in human extravillous trophoblasts(Cao, Papadopoulou et al. 2005, Wakahashi, Nakabayashi et al. 2008). CRHR1 Activation by CRH promotes intestinal inflammation, whereas CRHR2 activation by UCN3 inhibits the response(Im, Rhee et al. 2010). Furthermore, it was shown that CRH stimulates intestinal endothelial cells tube formation *in vitro*, enhances colitis-associated angiogenesis *in vivo* and increases aortic vessel outgrowth *ex vivo*. Conversely, UCN 3 decreases these activities. In addition, the inflamed intestine in CRHR1−/− mice had reduced microvascular density and VEGF-A concentrations, whereas the inflamed intestine of CRHR2−/− mice had enhanced angiogenesis and VEGF-A

concentration. CRH family of peptides up-regulates VEGF-A expression via activation of either CRHR1 or CRHR2 in the intestinal epithelial cells.

The underlying mechanisms of the VEGF-A synthesis include adenylyl cyclase activation and cAMP induction along with transcriptional CREB regulation. Thus, deficiency of CREB confers a protective effect in intestinal inflammation through reduced concentrations of VEGF-A. Some groups, however, showed that the CRH family of peptides decreased expression of VEGF. Activation of the either CRHR1 or CRHR2 signaling by CRH and UCN3, respectively increases VEGF-A levels in epithelial cells of intestines suggesting that these peptides could be a main player in inflammatory angiogenesis. VEGF expression is tightly regulated by factors such as hypoxia, cytokines, or growth factors (Forsythe, Jiang et al. 1996, Takahashi, Kureishi et al. 2002, Rhee, Ma et al. 2015). In response to hypoxia, HIF-1α regulate transcription of VEGF(Forsythe, Jiang et al. 1996) . However, recent studies indicate that HIF-1α regulates VEGF in hypoxia-independent settings as well (Isaacs, Jung et al. 2002, Im, Venkatakrishnan et al. 2005). Thus, it is further required to assess the effect of CRHRs on the functions of endothelial cells and to explore the underlying mechanisms of sFLT-1 and PLGF production such as p38 MAPK, key molecule involved in angiogenesis as well as HIF expression mediate CRH actions on sFLT-1/PLGF ratio as well as whether they are involved in CRH VEGF interactions.

The general consensus is that the principal cause of pre- eclampsia is insufficient placentation during the first trimester , despite the manifestation of preeclampsia in the second or third trimester. Many studies show that first- trimester sFLT-1 maternal concentrations in women who later develop preeclampsia are in excess than sFLT-1 concentrations in control women (Jacobs, Nassar et al. 2011) indicating that the elevation of release of sFLT-1 from the placenta begins in the first trimester. sFLT-1 is synthesized by placental trophoblasts and is secreted into the maternal circulation (Dimitrakova, Dimitrakov et al. 2004, Shibata, Rajakumar et al. 2005). sFLT-1 is an antagonist of VEGF and placental growth factor (PLGF) and may promote placental growth restriction and maternal endothelial damage(Karumanchi and Bdolah 2004). It has been shown that increased trophoblast release of sFLT-1 causes preeclampsia. Maynard et al. were the first to study that as a result of sFLT-1 administration, hypertension, edema

and proteinuria occurs in pregnant rats. Thus indicating that excess maternalsFLT-1 concentrations contributes to the pathogenesis of preeclampsia(Maynard, Min et al. 2003).

Given the significant role in the pathogenesis of preeclampsia, attempts to identify factors that regulate trophoblast sFLT-1 expression have been made so as to explore possible novel strategies for managing this condition. Until now, factors such as hypoxia , specific proteases and autoantibody for angiotensin receptor, have been demonstrated to increase trophoblast sFLT-1 secretion(Nagamatsu, Fujii et al. 2004, Zhou, Ahmad et al. 2008, Zhao, Gu et al. 2010). hypoxia induces expression of sFLT-1 in trophoblasts(Nagamatsu, Fujii et al. 2004) and that insufficient placentation, speculated to occur in the first trimester, increases synthesizes of sFLT-1 from placenta, and consequently systemic/local endothelial dysfunction, which may cause further placental hypoxia and sFLT-1 synthesizes; promoting a ‘‘vicious cycle(Karumanchi and Bdolah 2004) . Given that we have evidence from previous studies that CRH regulates angiogenesis, and that CRH regulates sFLT-1 PLGF in HUVECs, and that inadequate trophoblast invasion leading to impaired spiral artery remodeling is thought to be underlying pathogenesis of placental insufficiency syndrome, it is further investigated the regulation of sFLT-1 and PLGF molecules by CRH in HTR8 model.

In HTR8 cells, it has been demonstrated that hypoxia induces sFLT-1 and PLGF secretion, leading to increased sFLT-1 /PLGF ratio (approximately 15 at 6h, 25 at 24h which reaches saturation at 48h). It is further required to assess if hypoxia affects sFLT-1 and PLGF transcription in a time dependent manner. A time dependent release of PLGF is seen in high oxygen tension, although sFLT-1 release reaches a saturation at 48h. Hypoxia increases sFLT-1 release and PLGF release, but sFLT-1 release is more in comparison with PLGF release. Hypoxia increases VEGF mRNA and a trend towards decrease in PLGF mRNA is seen. It is further important to see the effect of hypoxia on sFLT-1 transcription and possibly it will increase. Overall, hypoxia increases sFLT-1 /PLGF ratio. CRH has no effect on sFLT-1 transcription under normal and low oxygen tension, but increases sFLT-1 release under low oxygen tension. Under altered oxygen tension, CRH increases VEGF mRNA more compared to PLGF mRNA. A trend towards an increase in PLGF release by CRH is seen. In the present study, we have shown that CRH is another factor that regulates sFLT-1 , PLGF molecules in

trophoblasts. Overall, CRH possibly favors angiogenic state under low oxygen tension at the transcription level, although CRH appears to increase sFLT-1/PLGF ratio at protein secretion level. There is no VEGF CRH interactions as seen in HUVEC. Hypoxia increases CRHR1 and decreases CRHR2 transcription. Possibly increased CRHR1 /CRHR2 ratio is more favoring anti angiogenic state under altered oxygen tension. CRH decreases transcription at CRHR1 and CRHR2 mRNA under high oxygen tension. CRH reduces hypoxia induced upregulation of CRHR1 but not CRHR2 mRNA. Thus, the reduction of CRHR1 mRNA in hypoxia by CRH may be important for CRH to favor angiogenic state and reduce sFLT-1 /PLGF ratio and reduce the biological effects of sFLT-1 on trophoblasts. There is a possibility that CRHR1 and CRHR2 has different roles to execute in HTR8, BEWO and HUVECs and overall, in trophoblast and endothelial cells.

8 Conclusion

CRH inhibits sFLT-1 /PLGF ratio in HUVECs under high oxygen tension. CRH regulates VEGF mediated sFLT-1 and PLGF in HUVECs. In HTR8 cells, CRH possibly favors angiogenic state under low oxygen tension at the transcription level, although CRH appears to increase sFLT-1/PLGF ratio at protein secretion level. Hypoxia increases CRHR1 and decreases CRHR2 transcription. CRH reduces hypoxia induced upregulation of CRHR1 but not CRHR2 mRNA. In conclusion, in these cell lines, it is possible to replicate in vivo setting which shows hypoxia is associated with secretion of anti angiogenic proteins.

CRH was unable reverse hypoxia mediated impaired fusogenic machinery and biochemical differentiation in BEWO cells and unable to restore the syncytialization. CRHRs are differentially regulated in syncytialised BEWO cells under altered oxygen tension. CRH blocks upregulation of CRHR1 gene expression in differentiated BEWO cells under high oxygen tension. CRH blocks CRHR1 in undifferentiated BEWO cells under low oxygen tension. CRHR1 is down regulated in syncytialised BEWO cells when exposed to altered oxygen tension. CRH when added to differentiating cells under low oxygen tension has no effect on its receptors. Moreover, sFLT-1 rich media mediates fusogenic machinery and under low oxygen tension. CRH further impairs this and CRHR1 is down regulated which is reversed under low oxygen tension.

In future, the aim is to

- Assess the regulation of SFLT-1 and PLGF at transcriptional, translational and secretion level under altered oxygen tension in HUVEC as well as HTR8.
- Confirm the involvement of CRHRs in regulation of angiogenic and anti angiogenic factors by using antagonists and / or sIRNA in different cell lines
- To confirm the above findings in placental explants
- To do functional studies – trophoblast invasion, migration, proliferation as well proliferation, migration, tube formation in HUVECS
- To assess if CRH reverses the effect of altered oxygen tension on morphological differentiation, syncytin protein expression in BEWO.

9 References

A. Hein, M. V. D. G. (2012). "Regulation of trophoblast 11β-hydroxysteroid dehydrogenase type 2 expression and role of corticotropin-releasing hormone." Endocrine Abstracts **29**.

Abrahams, V. M. and G. Mor (2005). "Toll-like receptors and their role in the trophoblast." Placenta **26**(7): 540-547.

Abrahams, V. M., S. L. Straszewski-Chavez, S. Guller and G. Mor (2004). "First trimester trophoblast cells secrete Fas ligand which induces immune cell apoptosis." Mol Hum Reprod **10**(1): 55-63.

Adams, R. H., A. Porras, G. Alonso, M. Jones, K. Vintersten, S. Panelli, A. Valladares, L. Perez, R. Klein and A. R. Nebreda (2000). "Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development." Mol Cell **6**(1): 109-116.

Ades, E. W., F. J. Candal, R. A. Swerlick, V. G. George, S. Summers, D. C. Bosse and T. J. Lawley (1992). "HMEC-1: establishment of an immortalized human microvascular endothelial cell line." J Invest Dermatol **99**(6): 683-690.

Agelaki, S., C. Tsatsanis, A. Gravanis and A. N. Margioris (2002). "Corticotropin-releasing hormone augments proinflammatory cytokine production from macrophages in vitro and in lipopolysaccharide-induced endotoxin shock in mice." Infect Immun **70**(11): 6068-6074.

Aggelidou, E., E. W. Hillhouse and D. K. Grammatopoulos (2002). "Up-regulation of nitric oxide synthase and modulation of the guanylate cyclase activity by corticotropin-releasing hormone but not urocortin II or urocortin III in cultured human pregnant myometrial cells." Proc Natl Acad Sci U S A **99**(5): 3300-3305.

Aguilera, G., M. Nikodemova, P. C. Wynn and K. J. Catt (2004). "Corticotropin releasing hormone receptors: two decades later." Peptides **25**(3): 319-329.

Ahmad, S. and A. Ahmed (2004). "Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia." Circ Res **95**(9): 884-891.

Ahmad, S., P. W. Hewett, B. Al-Ani, S. Sissaoui, T. Fujisawa, M. J. Cudmore and A. Ahmed (2011). "Autocrine activity of soluble FLT-1 controls endothelial cell function and angiogenesis." Vasc Cell **3**(1): 15.

Ahmed, A., C. Dunk, S. Ahmad and A. Khaliq (2000). "Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble FLT-1 by oxygen--a review." Placenta **21 Suppl A**: S16-24.

Ahmed, A., C. Dunk, D. Kniss and M. Wilkes (1997). "Role of VEGF receptor-1 (FLT-1) in mediating calcium-dependent nitric oxide release and limiting DNA synthesis in human trophoblast cells." Lab Invest **76**(6): 779-791.

Akolekar, R., A. Syngelaki, R. Sarquis, M. Zvanca and K. H. Nicolaides (2011). "Prediction of early, intermediate and late pre-eclampsia from maternalfactors, biophysical and biochemical markers at 11-13 weeks." Prenat Diagn **31**(1): 66-74.

Al-Nasiry, S., B. Spitz, M. Hanssens, C. Luyten and R. Pijnenborg (2006). "Differential effects of inducers of syncytialization and apoptosis on BEWO and JEG-3 choriocarcinoma cells." Hum Reprod **21**(1): 193-201.

Alessi, D. R., S. R. James, C. P. Downes, A. B. Holmes, P. R. Gaffney, C. B. Reese and P. Cohen (1997). "Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha." Curr Biol **7**(4): 261-269.

Ali, K. Z. M. (1997). "Stereological study of the effect of altitude on the trophoblast cell populations of human term placental villi." Placenta **18**(5): 447-450.
Allan, B. B. and W. E. Balch (1999). "Protein Sorting by Directed Maturation of Golgi Compartments." Science **285**(5424): 63-66.
Alsat, E., P. Wyplosz, A. Malassine, J. Guibourdenche, D. Porquet, C. Nessmann and D. Evain-Brion (1996). "Hypoxia impairs cell fusion and differentiation process in human cytotrophoblast, in vitro." J Cell Physiol **168**(2): 346-353.
Ambati, B. K., M. Nozaki, N. Singh, A. Takeda, P. D. Jani, T. Suthar, R. J. Albuquerque, E. Richter, E. Sakurai, M. T. Newcomb, M. E. Kleinman, R. B. Caldwell, Q. Lin, Y. Ogura, A. Orecchia, D. A. Samuelson, D. W. Agnew, J. St Leger, W. R. Green, P. J. Mahasreshti, D. T. Curiel, D. Kwan, H. Marsh, S. Ikeda, L. J. Leiper, J. M. Collinson, S. Bogdanovich, T. S. Khurana, M. Shibuya, M. E. Baldwin, N. Ferrara, H. P. Gerber, S. De Falco, J. Witta, J. Z. Baffi, B. J. Raisler and J. Ambati (2006). "Corneal avascularity is due to soluble VEGF receptor-1." Nature **443**(7114): 993-997.
Andersen, B., A. Milsted, G. Kennedy and J. H. Nilson (1988). "Cyclic AMP and phorbol esters interact synergistically to regulate expression of the chorionic gonadotropin genes." J Biol Chem **263**(30): 15578-15583.
Anin, S. A., G. Vince and S. Quenby (2004). "Trophoblast invasion." Hum Fertil (Camb) **7**(3): 169-174.
Anna Caroline, H., V. Manu, K. Tomoshige, G. Dimitris and H. D. Alan (2014). Novel Role of the Enzyme 11beta-Hydroxysteroid Dehydrogenase Type 2 (11?-HSD2) on Trophoblast Biology. Female Reproductive Endocrinology: Pregnancy, Endocrine Society**:** SAT-0076-SAT-0076.
Anna Caroline Hein1, M. V., Tomoshige Kino1, Dimitris Grammatopoulos3 and Alan H DeCherney4 (2014). "Novel Role of the Enzyme 11beta-Hydroxysteroid Dehydrogenase Type 2 (11β-HSD2) on Trophoblast Biology." Endocrine Society's 96th Annual Meeting and Expo, June 21–24, 2014 - Chicago.
Anton, P. M., J. Gay, A. Mykoniatis, A. Pan, M. O'Brien, D. Brown, K. Karalis and C. Pothoulakis (2004). "Corticotropin-releasing hormone (CRH) requirement in Clostridium difficile toxin A-mediated intestinal inflammation." Proceedings of the National Academy of Sciences of the United States of America **101**(22): 8503-8508.
Aplin, J. D. (1991). "Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro." J Cell Sci **99 (Pt 4)**: 681-692.
Aplin, J. D., T. Haigh, C. J. Jones, H. J. Church and L. Vicovac (1999). "Development of cytotrophoblast columns from explanted first-trimester human placental villi: role of fibronectin and integrin alpha5beta1." Biol Reprod **60**(4): 828-838.
Arbiser, J. L., K. Karalis, A. Viswanathan, C. Koike, B. Anand-Apte, E. Flynn, B. Zetter and J. A. Majzoub (1999). "Corticotropin-Releasing Hormone Stimulates Angiogenesis and Epithelial Tumor Growth in the Skin." JouRNAl of Investigative Dermatology **113**(5): 838-842.
Arbiser, J. L., C. C. Morton, G. A. Bruns and J. A. Majzoub (1988). "Human corticotropin releasing hormone gene is located on the long arm of chromosome 8." Cytogenet Cell Genet **47**(3): 113-116.
Ardati, A., V. Goetschy, J. Gottowick, S. Henriot, O. Valdenaire, U. Deuschle and G. J. Kilpatrick (1999). "Human CRF2 alpha and beta splice variants: pharmacological characterization using radioligand binding and a luciferase gene expression assay." Neuropharmacology **38**(3): 441-448.
Arroyo, J., M. Price, S. Straszewski-Chavez, R. J. Torry, G. Mor and D. S. Torry (2014). "XIAP protein is induced by placenta growth factor (PLGF) and decreased during preeclampsia in trophoblast cells." Syst Biol Reprod Med **60**(5): 263-273.

Arroyo, J. A., L. D. Brown and H. L. Galan (2009). "Placental MTOR and related signaling pathways in an Ovine Model of Intrauterine Growth Restriction (IUGR)." American jouRNAl of obstetrics and gynecology **201**(6): 616.e611-616.e617.
Athanassiades, A. and P. K. Lala (1998). "Role of placenta growth factor (PIGF) in human extravillous trophoblast proliferation, migration and invasiveness." Placenta **19**(7): 465-473.
Audhya, T., C. S. Hollander, D. H. Schlesinger and B. Hutchinson (1989). "Structural characterization and localization of corticotropin-releasing factor in testis." Biochim Biophys Acta **995**(1): 10-16.
Aufdenblatten, M., M. Baumann, L. Raio, B. Dick, B. M. Frey, H. Schneider, D. Surbek, B. Hocher and M. G. Mohaupt (2009). "Prematurity is related to high placental cortisol in preeclampsia." Pediatr Res **65**(2): 198-202.
Bailey, M. A., J. J. Mullins and C. J. Kenyon (2009). "Mineralocorticoid and Glucocorticoid Receptors Stimulate Epithelial Sodium Channel Activity in a Mouse Model of Cushing Syndrome." Hypertension **54**(4): 890-896.
Balcazar Morales, N. and C. Aguilar de Plata (2012). "Role of AKT/MTORC1 pathway in pancreatic β-cell proliferation." Colombia Médica : CM **43**(3): 235-243.
Balch, W. E., J. M. McCaffery, H. Plutner and M. G. Farquhar (1994). "Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum." Cell **76**(5): 841-852.
Bale, T. L., A. Contarino, G. W. Smith, R. Chan, L. H. Gold, P. E. Sawchenko, G. F. Koob, W. W. Vale and K. F. Lee (2000). "Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress." Nat Genet **24**(4): 410-414.
Bale, T. L., F. J. Giordano, R. P. Hickey, Y. Huang, A. K. Nath, K. L. Peterson, W. W. Vale and K.-F. Lee (2002). "Corticotropin-releasing factor receptor 2 is a tonic suppressor of vascularization." Proceedings of the National Academy of Sciences **99**(11): 7734-7739.
Bale, T. L., F. J. Giordano, R. P. Hickey, Y. Huang, A. K. Nath, K. L. Peterson, W. W. Vale and K. F. Lee (2002). "Corticotropin-releasing factor receptor 2 is a tonic suppressor of vascularization." Proc Natl Acad Sci U S A **99**(11): 7734-7739.
Bale, T. L., F. J. Giordano and W. W. Vale (2003). "A New Role for Corticotropin-Releasing Factor Receptor-2: Suppression of Vascularization." Trends in Cardiovascular Medicine **13**(2): 68-71.
Bamberger, A. M., V. Minas, S. N. Kalantaridou, J. Radde, H. Sadeghian, T. Loning, I. Charalampopoulos, J. Brummer, C. Wagener, C. M. Bamberger, H. M. Schulte, G. P. Chrousos and A. Makrigiannakis (2006). "Corticotropin-releasing hormone modulates human trophoblast invasion through carcinoembryonic antigen-related cell adhesion molecule-1 regulation." Am J Pathol **168**(1): 141-150.
Barlowe, C. (2003). "Signals for COPII-dependent export from the ER: what's the ticket out?" Trends in Cell Biology **13**(6): 295-300.
Barnes, P. J. (2006). "Receptor heterodimerization: a new level of cross-talk." JouRNAl of Clinical Investigation **116**(5): 1210-1212.
Beaudoin, C., C. H. Blomquist and Y. Tremblay (1995). "Gene expression of 17 beta-hydroxysteroid dehydrogenase type 2 isozyme in primary cultures of human trophoblasts predicts different mechanisms regulating type 1 and type 2 enzymes." Endocrinology **136**(9): 3807-3814.
Behan, D. P., O. Khongsaly, N. Ling and E. B. De Souza (1996). "Urocortin interaction with corticotropin-releasing factor (CRF) binding protein (CRF-BP): a novel mechanism for elevating "free' CRF levels in human brain." Brain Res **725**(2): 263-267.

Beitins, I. Z., F. Bayard, I. G. Ances, A. Kowarski and C. J. Migeon (1973). "The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term." Pediatr Res **7**(5): 509-519.
Benaitreau, D., E. D. Santos, M.-C. Leneveu, P. De Mazancourt, R. Pecquery and M.-N. Dieudonné (2010). "Adiponectin promotes syncytialisation of BEWO cell line and primary trophoblast cells." Reproductive Biology and Endocrinology **8**(1): 128.
Benirschke, K., P. Kaufmann, R. Baergen, K. Benirschke, P. Kaufmann and R. Baergen (2006). Pathology of the Human Placenta, Springer.
Benyo, D. F., T. M. Miles and K. P. Conrad (1997). "Hypoxia stimulates cytokine production by villous explants from the human placenta." J Clin Endocrinol Metab **82**(5): 1582-1588.
Berga, S. L., J. F. Nitsche and G. D. Braunstein (2016). Chapter 21 - Endocrine Changes in Pregnancy. Williams Textbook of Endocrinology (Thirteenth Edition). S. Melmed, K. S. Polonsky, P. R. Larsen and H. M. Kronenberg. Philadelphia, Content Repository Only!**:** 831-848.
BERKowitz, G. S., R. H. Lapinski, C. J. Lockwood, P. Florio, C. Blackmore-Prince and F. Petraglia (1996). "Corticotropin-releasing factor and its binding protein: maternalserum levels in term and preterm deliveries." Am J Obstet Gynecol **174**(5): 1477-1483.
Berra, E., G. Pagès and J. Pouysségur (2000). "MAP Kinases and Hypoxia in the Control of VEGF Expression." Cancer and Metastasis Reviews **19**(1): 139-145.
Berra, E., D. Roux, D. E. Richard and J. Pouysségur (2001). "Hypoxia-inducible factor-1α (HIF-1α) escapes O(2)-driven proteasomal degradation irrespective of its subcellular localization: nucleus or cytoplasm." EMBO Reports **2**(7): 615-620.
Binder, N. K., J. Evans, L. A. Salamonsen, D. K. Gardner, T. J. Kaitu'u-Lino and N. J. Hannan (2016). "Placental Growth Factor Is Secreted by the Human Endometrium and Has Potential Important Functions during Embryo Development and Implantation." PLoS One **11**(10): e0163096.
Bittencourt, J. C., J. Vaughan, C. Arias, R. A. Rissman, W. W. Vale and P. E. Sawchenko (1999). "Urocortin expression in rat brain: evidence against a pervasive relationship of urocortin-containing projections with targets bearing type 2 CRF receptors." J Comp Neurol **415**(3): 285-312.
Bloch, M., R. C. Daly and D. R. Rubinow (2003). "Endocrine factors in the etiology of postpartum depression." Compr Psychiatry **44**(3): 234-246.
Blond, J. L., D. Lavillette, V. Cheynet, O. Bouton, G. Oriol, S. Chapel-FeRNAndes, B. Mandrand, F. Mallet and F. L. Cosset (2000). "An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor." J Virol **74**(7): 3321-3329.
Boeckel, J.-N., V. Guarani, M. Koyanagi, T. Roexe, A. Lengeling, R. T. Schermuly, P. Gellert, T. Braun, A. Zeiher and S. Dimmeler (2011). "Jumonji domain-containing protein 6 (Jmjd6) is required for angiogenic sprouting and regulates splicing of VEGF-receptor 1." Proceedings of the National Academy of Sciences of the United States of America **108**(8): 3276-3281.
Boorse, G. C., E. J. Crespi, F. M. Dautzenberg and R. J. Denver (2005). "Urocortins of the South African clawed frog, Xenopus laevis: conservation of structure and function in tetrapod evolution." Endocrinology **146**(11): 4851-4860.
Bossi, G. and G. M. Griffiths (1999). "Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells." Nat Med **5**(1): 90-96.
Boynton, A. L. (1983). "The role of cyclic AMP in cell proliferation ; a critical assessment of the evidence." Adv. Cycl. Nucl. Res **15**: 193-294.
Brar, B. K., A. Chen, M. H. Perrin and W. Vale (2004). "Specificity and regulation of extracellularly regulated kinase1/2 phosphorylation through corticotropin-releasing factor

(CRF) receptors 1 and 2beta by the CRF/urocortin family of peptides." Endocrinology **145**(4): 1718-1729.
Breier, G., U. Albrecht, S. Sterrer and W. Risau (1992). "Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation." Development **114**(2): 521-532.
Brown, L. F., K. T. Yeo, B. Berse, T. K. Yeo, D. R. Senger, H. F. Dvorak and L. van de Water (1992). "Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing." The JouRNAl of Experimental Medicine **176**(5): 1375-1379.
Bukowski, R., G. D. Hankins, G. R. Saade, G. D. Anderson and S. Thornton (2006). "Labor-associated gene expression in the human uterine fundus, lower segment, and cervix." PLoS Med **3**(6): e169.
Burke, S. D., V. F. Barrette, J. Bianco, J. G. Thorne, A. T. Yamada, S. C. Pang, M. A. Adams and B. A. Croy (2010). "Spiral arterial remodeling is not essential for normal blood pressure regulation in pregnant mice." Hypertension **55**(3): 729-737.
Burton, G., S. Tham, G. Burton and S. Tham (1992). "The formation of vasculo-syncytial membranes in the human placenta." JouRNAl of Developmental Physiology **18**: 43-47.
Burton, G. J., D. S. Charnock-Jones and E. Jauniaux (2009). "Regulation of vascular growth and function in the human placenta." **138**(6): 895.
Burton, G. J., D. S. Charnock-Jones and E. Jauniaux (2009). "Regulation of vascular growth and function in the human placenta." Reproduction **138**(6): 895-902.
Burton, G. J. and E. Jauniaux (1995). "Sonographic, stereological and Doppler flow velocimetric assessments of placental maturity." Br J Obstet Gynaecol **102**(10): 818-825.
Burton, G. J., E. Jauniaux and A. L. Watson (1999). "Maternalarterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited." Am J Obstet Gynecol **181**(3): 718-724.
Burton, G. J., A. W. Woods, E. Jauniaux and J. C. Kingdom (2009). "Rheological and physiological consequences of conversion of the maternalspiral arteries for uteroplacental blood flow during human pregnancy." Placenta **30**(6): 473-482.
Bussolati, B., C. Dunk, M. Grohman, C. D. Kontos, J. Mason and A. Ahmed (2001). "Vascular endothelial growth factor receptor-1 modulates vascular endothelial growth factor-mediated angiogenesis via nitric oxide." Am J Pathol **159**(3): 993-1008.
Butler, T. M., P. A. Elustondo, G. E. Hannigan and D. J. MacPhee (2009). "Integrin-linked kinase can facilitate syncytialization and hormonal differentiation of the human trophoblast-derived BEWO cell line." Reproductive Biology and Endocrinology **7**(1): 51.
Caniggia, I., H. Mostachfi, J. Winter, M. Gassmann, S. J. Lye, M. Kuliszewski and M. Post (2000). "Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3)." J Clin Invest **105**(5): 577-587.
Caniggia, I., H. Mostachfi, J. Winter, M. Gassmann, S. J. Lye, M. Kuliszewski and M. Post (2000). "Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFβ(3)." JouRNAl of Clinical Investigation **105**(5): 577-587.
Cantarella, G., L. Lempereur, G. Lombardo, A. Chiarenza, C. Pafumi, G. Zappalà and R. BeRNArdini (2001). "Divergent effects of corticotropin releasing hormone on endothelial cell nitric oxide synthase are associated with different expression of CRH type 1 and 2 receptors." British JouRNAl of Pharmacology **134**(4): 837-844.
Cao, J., N. Papadopoulou, D. Kempuraj, W. S. Boucher, K. Sugimoto, C. L. Cetrulo and T. C. Theoharides (2005). "Human Mast Cells Express Corticotropin-Releasing Hormone (CRH) Receptors and CRH Leads to Selective Secretion of Vascular Endothelial Growth Factor." The JouRNAl of Immunology **174**(12): 7665-7675.

Cargnello, M. and P. P. Roux (2011). "Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases." Microbiol Mol Biol Rev **75**(1): 50-83.
Carmeliet, P. (2000). "Mechanisms of angiogenesis and arteriogenesis." Nat Med **6**(4): 389-395.
Carmeliet, P. (2000). "VEGF gene therapy: stimulating angiogenesis or angioma-genesis?" Nat Med **6**(10): 1102-1103.
Carmeliet, P. (2003). "Angiogenesis in health and disease." Nat Med **9**(6): 653-660.
Carmeliet, P., Y. Dor, J. M. Herbert, D. Fukumura, K. Brusselmans, M. Dewerchin, M. Neeman, F. Bono, R. Abramovitch, P. Maxwell, C. J. Koch, P. Ratcliffe, L. Moons, R. K. Jain, D. Collen and E. Keshert (1998). "Role of HIF-1 alphalpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis." Nature **394**(6692): 485-490.
Carsten, M. E. and J. D. Miller (1985). "Ca2+ release by inositol trisphosphate from Ca2+-transporting microsomes derived from uterine sarcoplasmic reticulum." Biochem Biophys Res Commun **130**(3): 1027-1031.
Carter, A. M. (2000). "Placental oxygen consumption. Part I: in vivo studies--a review." Placenta **21 Suppl A**: S31-37.
Cass, L. A., S. A. Summers, G. V. Prendergast, J. M. Backer, M. J. Birnbaum and J. L. Meinkoth (1999). "Protein Kinase A-Dependent and -Independent Signaling Pathways Contribute to Cyclic AMP-Stimulated Proliferation." Molecular and Cellular Biology **19**(9): 5882-5891.
Catalano, R. D., T. Kyriakou, J. Chen, A. Easton and E. W. Hillhouse (2003). "Regulation of corticotropin-releasing hormone type 2 receptors by multiple promoters and alteRNAtive splicing: identification of multiple splice variants." Mol Endocrinol **17**(3): 395-410.
Chan, E. C., M. Thomson, G. Madsen, J. Falconer and R. Smith (1988). "Differential processing of corticotrophin-releasing hormone by the human placenta and hypothalamus." Biochem Biophys Res Commun **153**(3): 1229-1235.
Chang, C. P., R. V. Pearse, 2nd, S. O'Connell and M. G. Rosenfeld (1993). "Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain." Neuron **11**(6): 1187-1195.
Chappell, L. C., S. Duckworth, P. T. Seed, M. Griffin, J. Myers, L. Mackillop, N. Simpson, J. Waugh, D. Anumba, L. C. Kenny, C. W. Redman and A. H. Shennan (2013). "Diagnostic accuracy of placental growth factor in women with suspected preeclampsia: a prospective multicenter study." Circulation **128**(19): 2121-2131.
Chasin, L. A. (2007). "Searching for splicing motifs." Adv Exp Med Biol **623**: 85-106.
Chatzaki, E., A. Makrigiannakis, A. N. Margioris, E. Kouimtzoglou and A. Gravanis (2001). "The Fas/FasL apoptotic pathway is involved in kappa-opioid-induced apoptosis of human endometrial stromal cells." Mol Hum Reprod **7**(9): 867-874.
Chau, K., A. Hennessy and A. Makris (2017). "Placental growth factor and pre-eclampsia." JouRNAl of Human Hypertension **31**(12): 782-786.
Chen, A., E. Zorrilla, S. Smith, D. Rousso, C. Levy, J. Vaughan, C. Donaldson, A. Roberts, K. F. Lee and W. Vale (2006). "Urocortin 2-deficient mice exhibit gender-specific alterations in circadian hypothalamus-pituitary-adrenal axis and depressive-like behavior." J Neurosci **26**(20): 5500-5510.
Chen, D.-b. and J. Zheng (2014). "Regulation of Placental Angiogenesis." Microcirculation (New York, N.Y. : 1994) **21**(1): 15-25.
Chen, J. X., M. L. Lawrence, G. Cunningham, B. W. Christman and B. Meyrick (2004). "HSP90 and AKT modulate Ang-1-induced angiogenesis via NO in coronary artery endothelium." J Appl Physiol (1985) **96**(2): 612-620.
Chen, R., K. A. Lewis, M. H. Perrin and W. W. Vale (1993). "Expression cloning of a human corticotropin-releasing-factor receptor." Proc Natl Acad Sci U S A **90**(19): 8967-8971.

Chen, W. S., P. Z. Xu, K. Gottlob, M. L. Chen, K. Sokol, T. Shiyanova, I. Roninson, W. Weng, R. Suzuki, K. Tobe, T. Kadowaki and N. Hay (2001). "Growth retardation and increased apoptosis in mice with homozygous disruption of the AKT1 gene." Genes Dev **15**(17): 2203-2208.
Chen, Y., M. Allars, X. Pan, K. Maiti, G. Angeli, R. Smith and R. C Nicholson (2013). Effects of corticotrophin releasing hormone (CRH) on cell viability and differentiation in the human BEWO choriocarcinoma cell line: A potential syncytialisation inducer distinct from cyclic adenosine monophosphate (cAMP).
Chiang, M., F. Liang, C. Chen, C. Chang, M. Cheong, L. Wang, C. Liang, F. Lin, C. Chou, H. Chen, M. Chiang, F. Liang, C. Chen, C. Chang, M. Cheong, L. Wang, C. Liang, F. Lin, C. Chou and H. Chen (2009). "Mechanism of hypoxia-induced GCM1 degradation: implications for the pathogenesis of preeclampsia." JouRNAl of Biological Chemistry **284**: 17411-17419.
Cho, H., J. L. Thorvaldsen, Q. Chu, F. Feng and M. J. Birnbaum (2001). "AKT1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice." J Biol Chem **276**(42): 38349-38352.
Choi, S. B., J. B. Park, T. J. Song and S. Y. Choi (2011). "Molecular mechanism of HIF-1-independent VEGF expression in a hepatocellular carcinoma cell line." Int J Mol Med **28**(3): 449-454.
Chou, J. Y. (1978). "Human placental cells transformed by tsA mutants of simian virus 40: a model system for the study of placental functions." Proceedings of the National Academy of Sciences **75**(3): 1409-1413.
Cindrova-Davies, T., H. Yung, J. Johns, O. Spasic-Boskovic, S. Korolchuk, E. Jauniaux, G. Burton, D. Charnock-Jones, T. Cindrova-Davies, H. Yung, J. Johns, O. Spasic-Boskovic, S. Korolchuk, E. Jauniaux, G. Burton and D. Charnock-Jones (2007). "Oxidative stress, gene expression, and protein changes induced in the human placenta during labor." American JouRNAl of Pathology **171**: 1168-1179.
Clark, D. E., S. K. Smith, Y. He, K. A. Day, D. R. Licence, A. N. Corps, R. Lammoglia and D. S. Charnock-Jones (1998). "A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternalcirculation." Biol Reprod **59**(6): 1540-1548.
Clauss, M., M. Gerlach, H. Gerlach, J. Brett, F. Wang, P. C. Familletti, Y. C. Pan, J. V. Olander, D. T. Connolly and D. Stern (1990). "Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration." J Exp Med **172**(6): 1535-1545.
Clauss, M., M. Gerlach, H. Gerlach, J. Brett, F. Wang, P. C. Familletti, Y. C. Pan, J. V. Olander, D. T. Connolly and D. Stern (1990). "Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration." The JouRNAl of Experimental Medicine **172**(6): 1535-1545.
Clifton, V. L., Q. Gu, V. E. Murphy, J. Schwartz, G. Madsen and R. Smith (2000). "Localization and characterization of urocortin during human pregnancy." Placenta **21**(8): 782-788.
Clifton, V. L., P. C. Owens, P. J. Robinson and R. Smith (1995). "Identification and characterization of a corticotrophin-releasing hormone receptor in human placenta." Eur J Endocrinol **133**(5): 591-597.
Clifton, V. L., M. A. Read, A. L. Boura, P. J. Robinson and R. Smith (1996). "Adrenocorticotropin causes vasodilatation in the human fetal-placental circulation." J Clin Endocrinol Metab **81**(4): 1406-1410.
Clifton, V. L., M. A. Read, I. M. Leitch, A. L. Boura, P. J. Robinson and R. Smith (1994). "Corticotropin-releasing hormone-induced vasodilatation in the human fetal placental circulation." J Clin Endocrinol Metab **79**(2): 666-669.

Clifton, V. L., M. A. Read, I. M. Leitch, W. B. Giles, A. L. Boura, P. J. Robinson and R. Smith (1995). "Corticotropin-releasing hormone-induced vasodilatation in the human fetal-placental circulation: involvement of the nitric oxide-cyclic guanosine 3',5'-monophosphate-mediated pathway." J Clin Endocrinol Metab **80**(10): 2888-2893.
Clifton, V. L., J. F. Telfer, A. J. Thompson, I. T. Cameron, T. G. Teoh, S. J. Lye and J. R. Challis (1998). "Corticotropin-releasing hormone and proopiomelanocortin-derived peptides are present in human myometrium." J Clin Endocrinol Metab **83**(10): 3716-3721.
Connors, B. W. (2012). "Tales of a dirty drug: carbenoxolone, gap junctions, and seizures." Epilepsy Curr **12**(2): 66-68.
Cope, J. L., L. Regev, Y. Chen, A. Korosi, C. J. Rice, S. Ji, G. A. Rogge, M. A. Wood and T. Z. Baram (2014). "Differential contribution of CBP:CREB binding to corticotropin-releasing hormone expression in the infant and adult hypothalamus." Stress **17**(1): 39-50.
Critchley, G. R. and G. J. Burton (1987). "Intralobular variations in barrier thickness in the mature human placenta." Placenta **8**(2): 185-194.
Crocker, I. P., B. K. Strachan, G. E. Lash, S. Cooper, A. Y. Warren and P. N. Baker (2001). "Vascular endothelial growth factor but not placental growth factor promotes trophoblast syncytialization in vitro." J Soc Gynecol Investig **8**(6): 341-346.
Cronier, L., B. Bastide, J. C. Herve, J. Deleze and A. Malassine (1994). "Gap junctional communication during human trophoblast differentiation: influence of human chorionic gonadotropin." Endocrinology **135**(1): 402-408.
Cross, D. A., D. R. Alessi, P. Cohen, M. Andjelkovich and B. A. Hemmings (1995). "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B." Nature **378**(6559): 785-789.
Cross, J. C. (2003). "The genetics of pre-eclampsia: a feto-placental or maternalproblem?" Clin Genet **64**(2): 96-103.
Cross, J. C., Z. Werb and S. J. Fisher (1994). "Implantation and the placenta: key pieces of the development puzzle." Science **266**(5190): 1508-1518.
Cudmore, M. J., P. W. Hewett, S. Ahmad, K.-Q. Wang, M. Cai, B. Al-Ani, T. Fujisawa, B. Ma, S. Sissaoui, W. Ramma, M. R. Miller, D. E. Newby, Y. Gu, B. Barleon, H. Weich and A. Ahmed (2012). "The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis." Nature Communications **3**: 972.
Daisuke, F., T. Akiko, S. Tatsuharu, S. Hekiko, N. Keijirou, Y. Yoshiki, T. Yoshito, K. Hideki and O. Masahide (2010). "Role of extracellular signal-regulated kinase and AKT cascades in regulating hypoxia-induced angiogenic factors produced by a trophoblast-derived cell line." JouRNAl of Endocrinology **206**(1): 131-140.
Dale, H. H. (1906). "On some physiological actions of ergot." J Physiol **34**(3): 163-206.
Dang, F., B. A. Croy, P. W. Stroman and E. A. Figueiro-Filho (2016). "Impacts of Preeclampsia on the Brain of the Offspring." Rev Bras Ginecol Obstet **38**(8): 416-422.
Daoud, G., M. Amyot, E. Rassart, A. Masse, L. Simoneau and J. Lafond (2005). "ERK1/2 and p38 regulate trophoblasts differentiation in human term placenta." J Physiol **566**(Pt 2): 409-423.
Daoud, G., M. Amyot, É. Rassart, A. Masse, L. Simoneau and J. Lafond (2005). "ERK1/2 and p38 regulate trophoblasts differentiation in human term placenta." The JouRNAl of Physiology **566**(Pt 2): 409-423.
Dashwood, M. R., H. E. Andrews and E. T. Wei (1987). "Binding of [125I]Tyr-corticotropin-releasing factor to rabbit aorta is reduced by removal of the endothelium." Eur J Pharmacol **135**(1): 111-112.
Dautzenberg, F. M. and R. L. Hauger (2002). "The CRF peptide family and their receptors: yet more partners discovered." Trends Pharmacol Sci **23**(2): 71-77.

Delidaki, M., M. Gu, A. Hein, M. Vatish and D. K. Grammatopoulos (2011). "Interplay of cAMP and MAPK pathways in hCG secretion and fusogenic gene expression in a trophoblast cell line." Mol Cell Endocrinol **332**(1-2): 213-220.
Depoix, C., M. K. Tee and R. N. Taylor (2011). "Molecular Regulation of Human Placental Growth Factor (PLGF) Gene Expression in Placental Villi and Trophoblast Cells is Mediated via the Protein Kinase A Pathway." Reproductive Sciences **18**(3): 219-228.
Deprez, J., D. Vertommen, D. R. Alessi, L. Hue and M. H. Rider (1997). "Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades." J Biol Chem **272**(28): 17269-17275.
Dermitzaki, E., C. Tsatsanis, A. Gravanis and A. N. Margioris (2002). "Corticotropin-releasing hormone induces Fas ligand production and apoptosis in PC12 cells via activation of p38 mitogen-activated protein kinase." J Biol Chem **277**(14): 12280-12287.
Derynck, R., J. A. Jarrett, E. Y. Chen, D. H. Eaton, J. R. Bell, R. K. Assoian, A. B. Roberts, M. B. Sporn and D. V. Goeddel (1985). "Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells." Nature **316**(6030): 701-705.
Desai, J., V. Holt-Shore, R. J. Torry, M. R. Caudle and D. S. Torry (1999). "Signal transduction and biological function of placenta growth factor in primary human trophoblast." Biol Reprod **60**(4): 887-892.
Dhar, R., S. Karmakar, R. Sriraman, G. Prasad Talwar and C. Das (2004). Efficacy of a Recombinant Chimeric Anti-hCG Antibody to Prevent Human Cytotrophoblasts Fusion and Block Progesterone Synthesis.
Dhar, R., S. Karmakar, R. Sriraman, G. P. Talwar and C. Das (2004). "Efficacy of a Recombinant Chimeric Anti-hCG Antibody to Prevent Human Cytotrophoblasts Fusion and Block Progesterone Synthesis." American JouRNAl of Reproductive Immunology **51**(5): 358-363.
Dieterich, K. D., H. Lehnert and E. B. De Souza (1997). "Corticotropin-releasing factor receptors: an overview." Exp Clin Endocrinol Diabetes **105**(2): 65-82.
Dimitrakova, E. D., J. D. Dimitrakov, S. A. Karumanchi, B. K. Pehlivanov, N. P. Milchev and D. I. Dimitrakov (2004). "Placental soluble fms-like tyrosine-kinase-1 (sFLT-1) in pregnant women with preeclampsia." Folia Med (Plovdiv) **46**(1): 19-21.
Domigan, C. K., C. M. Warren, V. Antanesian, K. Happel, S. Ziyad, S. Lee, A. Krall, L. Duan, A. X. Torres-Collado, L. W. Castellani, D. Elashoff, H. R. Christofk, A. M. van der Bliek, M. Potente and M. L. Iruela-Arispe (2015). "Autocrine VEGF maintains endothelial survival through regulation of metabolism and autophagy." JouRNAl of Cell Science **128**(12): 2236-2248.
Donaldson, C. J., S. W. Sutton, M. H. Perrin, A. Z. Corrigan, K. A. Lewis, J. E. Rivier, J. M. Vaughan and W. W. Vale (1996). "Cloning and characterization of human urocortin." Endocrinology **137**(5): 2167-2170.
Dumont, J. E., J. C. Jauniaux and P. P. Roger (1989). "The cyclic AMP-mediated stimulation of cell proliferation." Trends Biochem Sci **14**(2): 67-71.
Dunk, C. and A. Ahmed (2001). "Vascular Endothelial Growth Factor Receptor-2-Mediated Mitogenesis Is Negatively Regulated by Vascular Endothelial Growth Factor Receptor-1 in Tumor Epithelial Cells." The American JouRNAl of Pathology **158**(1): 265-273.
Easton, R. M., H. Cho, K. Roovers, D. W. Shineman, M. Mizrahi, M. S. Forman, V. M. Lee, M. Szabolcs, R. de Jong, T. Oltersdorf, T. Ludwig, A. Efstratiadis and M. J. Birnbaum (2005). "Role for AKT3/protein kinase Bgamma in attainment of normal brain size." Mol Cell Biol **25**(5): 1869-1878.

Economides, D., E. Linton, K. Nicolaides, C. H. Rodeck, P. J. Lowry and T. Chard (1987). "Relationship between maternaland fetal corticotrophin-releasing hormone-41 and ACTH levels in human mid-trimester pregnancy." J Endocrinol **114**(3): 497-501.
Erickson, K., P. Thorsen, G. Chrousos, D. E. Grigoriadis, O. Khongsaly, J. McGregor and J. Schulkin (2001). "Preterm birth: associated neuroendocrine, medical, and behavioral risk factors." J Clin Endocrinol Metab **86**(6): 2544-2552.
Esterman, A., T. H. Finlay and J. Dancis (1996). "The effect of hypoxia on term trophoblast: hormone synthesis and release." Placenta **17**(4): 217-222.
Europe-Finner, G. N., S. Phaneuf, A. M. Tolkovsky, S. P. Watson and A. Lopez BeRNAl (1994). "Down-regulation of G alpha s in human myometrium in term and preterm labor: a mechanism for parturition." J Clin Endocrinol Metab **79**(6): 1835-1839.
Evans, R. T. and A. F. Seasholtz (2009). "Soluble corticotropin-releasing hormone receptor 2alpha splice variant is efficiently translated but not trafficked for secretion." Endocrinology **150**(9): 4191-4202.
Fadhillah, S. Yoshioka, R. Nishimura and K. Okuda (2014). "Hypoxia Promotes Progesterone Synthesis During Luteinization in Bovine Granulosa Cells." The JouRNAl of Reproduction and Development **60**(3): 194-201.
Fahlbusch, F. B., A. Hartner, C. Menendez-Castro, S. C. Nogel, I. Marek, M. W. Beckmann, E. Schleussner, M. Ruebner, H. Huebner, H. G. Dorr, R. L. Schild, J. Dotsch and W. Rascher (2015). "The placental MTOR-pathway: correlation with early growth trajectories following intrauterine growth restriction?" J Dev Orig Health Dis **6**(4): 317-326.
Fahlbusch, F. B., M. Ruebner, G. Volkert, R. Offergeld, A. Hartner, C. Menendez-Castro, R. Strick, M. Rauh, W. Rascher and J. Dötsch (2012). "Corticotropin-releasing hormone stimulates expression of leptin, 11beta-HSD2 and syncytin-1 in primary human trophoblasts." Reproductive Biology and Endocrinology **10**(1): 80.
Fan, J. M., X. Q. Chen, H. Jin and J. Z. Du (2009). "Gestational hypoxia alone or combined with restraint sensitizes the hypothalamic-pituitary-adrenal axis and induces anxiety-like behavior in adult male rat offspring." Neuroscience **159**(4): 1363-1373.
Fang, H., Y.-J. Sun, Y.-H. Lv, R.-J. Ni, Y.-M. Shu, X.-Y. Feng, Y. Wang, Q.-H. Shan, Y.-N. Zu and J.-N. Zhou (2016). "High activity of the stress promoter contributes to susceptibility to stress in the tree shrew." Scientific Reports **6**: 24905.
Feng, L., W. X. Liao, Q. Luo, H. H. Zhang, W. Wang, J. Zheng and D. B. Chen (2012). "Caveolin-1 orchestrates fibroblast growth factor 2 signaling control of angiogenesis in placental artery endothelial cell caveolae." J Cell Physiol **227**(6): 2480-2491.
Feng, L., H. H. Zhang, W. Wang, J. Zheng and D. B. Chen (2012). "Compartmentalizing proximal FGFR1 signaling in ovine placental artery endothelial cell caveolae." Biol Reprod **87**(2): 40.
Ferrara, N., H. P. Gerber and J. LeCouter (2003). "The biology of VEGF and its receptors." Nat Med **9**(6): 669-676.
Filippa, N., C. L. Sable, C. Filloux, B. Hemmings and E. Van Obberghen (1999). "Mechanism of Protein Kinase B Activation by Cyclic AMP-Dependent Protein Kinase." Molecular and Cellular Biology **19**(7): 4989-5000.
Florio, P., F. Arcuri, P. Ciarmela, Y. Runci, R. Romagnoli, M. Cintorino, A. M. Di Blasio and F. Petraglia (2002). "Identification of urocortin mRNA and peptide in the human endometrium." J Endocrinol **173**(2): R9-14.
Florio, P., G. De Falco, E. Leucci, M. Torricelli, P. B. Torres, P. Toti, A. Dell'Anna, E. Tiso, R. Santopietro, L. Leoncini and F. Petraglia (2006). "Urocortin expression is downregulated in human endometrial carcinoma." J Endocrinol **190**(1): 99-105.

Florio, P., A. Franchini, F. M. Reis, I. Pezzani, E. Ottaviani and F. Petraglia (2000). "Human placenta, chorion, amnion and decidua express different variants of corticotropin-releasing factor receptor messenger RNA." Placenta **21**(1): 32-37.
Florio, P., P. J. Lowry, C. Benedetto, L. Galleri, M. Torricelli, A. Giovannelli, R. Battista, F. M. Reis and F. Petraglia (2007). "Maternalplasma corticotropin-releasing factor (CRF) and CRF-binding protein (CRF-BP) levels in post-term pregnancy: effect of prostaglandin administration." Eur J Endocrinol **157**(3): 279-284.
Florio, P., F. M. Severi, P. Ciarmela, G. Fiore, G. Calonaci, A. Merola, C. De Felice, M. Palumbo and F. Petraglia (2002). "Placental stress factors and maternal-fetal adaptive response: the corticotropin-releasing factor family." Endocrine **19**(1): 91-102.
Florio, P., M. Torricelli, G. De Falco, E. Leucci, A. Giovannelli, D. Gazzolo, F. M. Severi, F. Bagnoli, L. Leoncini, E. A. Linton and F. Petraglia (2006). "High maternaland fetal plasma urocortin levels in pregnancies complicated by hypertension." J Hypertens **24**(9): 1831-1840.
Florio, P., M. Torricelli, L. Galleri, G. De Falco, E. Leucci, G. Calonaci, E. Picciolini, G. Ambrosini, E. A. Linton and F. Petraglia (2005). "High fetal urocortin levels at term and preterm labor." J Clin Endocrinol Metab **90**(9): 5361-5365.
Florio, P., R. J. Woods, A. R. Genazzani, P. J. Lowry and F. Petraglia (1997). "Changes in amniotic fluid immunoreactive corticotropin-releasing factor (CRF) and CRF-binding protein levels in pregnant women at term and during labor." J Clin Endocrinol Metab **82**(3): 835-838.
Fogarty, N. M., T. M. Mayhew, A. C. Ferguson-Smith and G. J. Burton (2011). "A quantitative analysis of transcriptionally active syncytiotrophoblast nuclei across human gestation." J Anat **219**(5): 601-610.
Fong, G. H., J. Rossant, M. Gertsenstein and M. L. Breitman (1995). "Role of the FLT-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium." Nature **376**(6535): 66-70.
Förstermann, U., E. I. Closs, J. S. Pollock, M. Nakane, P. Schwarz, I. Gath and H. Kleinert (1994). "Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions." Hypertension **23**(6 Pt 2): 1121-1131.
Forsythe, J. A., B. H. Jiang, N. V. Iyer, F. Agani, S. W. Leung, R. D. Koos and G. L. Semenza (1996). "Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1." Molecular and Cellular Biology **16**(9): 4604-4613.
Forti, F. L. and H. A. Armelin (2000). "Acth Inhibits a Ras-Dependent Anti-Apoptotic and Mitogenic Pathway in Mouse Y1 Adrenocortical Cells." Endocrine Research **26**(4): 911-914.
Fox, H. (1964). "THE VILLOUS CYTOTROPHOBLAST AS AN INDEX OF PLACENTAL ISCHAEMIA." J Obstet Gynaecol Br Commonw **71**: 885-893.
Fox, H. (1967). "THE INCIDENCE AND SIGNIFICANCE OF VASCULO-SYNCYTIAL MEMBRANES IN THE HUMAN PLACENTA." BJOG: An InteRNAtional JouRNAl of Obstetrics & Gynaecology **74**(1): 28-33.
Frampton, G. K., J. Jones, M. Rose and L. Payne (2016). "Placental growth factor (alone or in combination with soluble fms-like tyrosine kinase 1) as an aid to the assessment of women with suspected pre-eclampsia: systematic review and economic analysis." Health Technol Assess **20**(87): 1-160.
Frendo, J. L., D. Olivier, V. Cheynet, J. L. Blond, O. Bouton, M. Vidaud, M. Rabreau, D. Evain-Brion and F. Mallet (2003). "Direct involvement of HERV-W Env glycoprotein in human trophoblast cell fusion and differentiation." Mol Cell Biol **23**(10): 3566-3574.
Frim, D. M., R. L. Emanuel, B. G. Robinson, C. M. Smas, G. K. Adler and J. A. Majzoub (1988). "Characterization and gestational regulation of corticotropin-releasing hormone messenger RNA in human placenta." J Clin Invest **82**(1): 287-292.
Fujita, D., A. Tanabe, T. Sekijima, H. Soen, K. Narahara, Y. Yamashita, Y. Terai, H. Kamegai and M. Ohmichi (2010). "Role of extracellular signal-regulated kinase and AKT

cascades in regulating hypoxia-induced angiogenic factors produced by a trophoblast-derived cell line." J Endocrinol **206**(1): 131-140.
Gaeggeler, H.-P., E. Gonzalez-Rodriguez, N. F. Jaeger, D. Loffing-Cueni, R. Norregaard, J. Loffing, J.-D. Horisberger and B. C. Rossier (2005). "Mineralocorticoid versus Glucocorticoid Receptor Occupancy Mediating Aldosterone-Stimulated Sodium Transport in a Novel Renal Cell Line." JouRNAl of the American Society of Nephrology **16**(4): 878-891.
Gamez, I., R. P. Ryan, L. D. Reid, S. M. Routt and B. A. Hollister (2011). "Corticorelin acetate, a synthetic corticotropin-releasing factor with preclinical antitumor activity, alone and with bevacizumab, against human solid tumor models." Cancer Chemotherapy and Pharmacology **67**(6): 1415-1422.
Gangestad, S. W., A. E. Caldwell Hooper and M. A. Eaton (2012). "On the function of placental corticotropin-releasing hormone: a role in maternal-fetal conflicts over blood glucose concentrations." Biological Reviews **87**(4): 856-873.
Gangloff, Y.-G., M. Mueller, S. G. Dann, P. Svoboda, M. Sticker, J.-F. Spetz, S. H. Um, E. J. Brown, S. Cereghini, G. Thomas and S. C. Kozma (2004). "Disruption of the Mouse MTOR Gene Leads to Early Postimplantation Lethality and Prohibits Embryonic Stem Cell Development." Molecular and Cellular Biology **24**(21): 9508-9516.
Gangloff, Y. G., M. Mueller, S. G. Dann, P. Svoboda, M. Sticker, J. F. Spetz, S. H. Um, E. J. Brown, S. Cereghini, G. Thomas and S. C. Kozma (2004). "Disruption of the mouse MTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development." Mol Cell Biol **24**(21): 9508-9516.
Gao, L., P. He, J. Sha, C. Liu, L. Dai, N. Hui and X. Ni (2007). "Corticotropin-releasing hormone receptor type 1 and type 2 mediate differential effects on 15-hydroxy prostaglandin dehydrogenase expression in cultured human chorion trophoblasts." Endocrinology **148**(8): 3645-3654.
Garcia-Lloret, M., D. Morrish, T. Wegmann, L. Honore, A. Turner, L. Guilbert, M. Garcia-Lloret, D. Morrish, T. Wegmann, L. Honore, A. Turner and L. Guilbert (1994). "Demonstration of functional cytokine–placental interactions: CSF-1 and GM-CSF stimulate human cytotrophoblast differentiation and peptide hormone secretion." Experimental Cell Research **214**: 46-54.
Garcia-Lloret, M. I., D. W. Morrish, T. G. Wegmann, L. Honore, A. R. Turner and L. J. Guilbert (1994). "Demonstration of functional cytokine-placental interactions: CSF-1 and GM-CSF stimulate human cytotrophoblast differentiation and peptide hormone secretion." Exp Cell Res **214**(1): 46-54.
Gaszner, B., A. Korosi, M. Palkovits, E. W. Roubos and T. Kozicz (2007). "Neuropeptide Y activates urocortin 1 neurons in the nonpreganglionic Edinger-Westphal nucleus." J Comp Neurol **500**(4): 708-719.
Gauster, M., G. Moser, K. Orendi and B. Huppertz (2009). "Factors involved in regulating trophoblast fusion: potential role in the development of preeclampsia." Placenta **30 Suppl A**: S49-54.
Genbacev, O., R. Joslin, C. H. Damsky, B. M. Polliotti and S. J. Fisher (1996). "Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia." J Clin Invest **97**(2): 540-550.
Genbacev, O., Y. Zhou, J. W. Ludlow and S. J. Fisher (1997). "Regulation of human placental development by oxygen tension." Science **277**(5332): 1669-1672.
Gennari-Moser, C., E. V. Khankin, S. Schuller, G. Escher, B. M. Frey, C. B. Portmann, M. U. Baumann, A. D. Lehmann, D. Surbek, S. A. Karumanchi, F. J. Frey and M. G. Mohaupt (2011). "Regulation of placental growth by aldosterone and cortisol." Endocrinology **152**(1): 263-271.

George, E. M., D. Colson, J. Dixon, A. C. Palei and J. P. Granger (2012). "Heme Oxygenase-1 Attenuates Hypoxia-Induced sFLT-1 and Oxidative Stress in Placental Villi through Its Metabolic Products CO and Bilirubin." InteRNAtional JouRNAl of Hypertension **2012**: 486053.
Gerber, H. P., F. Condorelli, J. Park and N. Ferrara (1997). "Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. FLT-1, but not Flk-1/KDR, is up-regulated by hypoxia." J Biol Chem **272**(38): 23659-23667.
Gerber, H. P., A. McMurtrey, J. Kowalski, M. Yan, B. A. Keyt, V. Dixit and N. Ferrara (1998). "Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/AKT signal transduction pathway. Requirement for Flk-1/KDR activation." J Biol Chem **273**(46): 30336-30343.
Gibb, W. and J. R. Challis (2002). "Mechanisms of term and preterm birth." J Obstet Gynaecol Can **24**(11): 874-883.
Glick, B. S. and V. Malhotra (1998). "The Curious Status of the Golgi Apparatus." Cell **95**(7): 883-889.
Glynn, B. P., A. Wolton, B. Rodriguez-Linares, S. Phaneuf and E. A. Linton (1998). "Urocortin in pregnancy." Am J Obstet Gynecol **179**(2): 533-539.
Gobble, R. M., K. A. Groesch, M. Chang, R. J. Torry and D. S. Torry (2009). "Differential regulation of human PLGF gene expression in trophoblast and nontrophoblast cells by oxygen tension." Placenta **30**(10): 869-875.
Goland, R. S., S. Jozak, W. B. Warren, I. M. Conwell, R. I. Stark and P. J. Tropper (1993). "Elevated levels of umbilical cord plasma corticotropin-releasing hormone in growth-retarded fetuses." J Clin Endocrinol Metab **77**(5): 1174-1179.
Goland, R. S., S. L. Wardlaw, R. I. Stark, L. S. Brown, Jr. and A. G. Frantz (1986). "High levels of corticotropin-releasing hormone immunoactivity in maternaland fetal plasma during pregnancy." J Clin Endocrinol Metab **63**(5): 1199-1203.
Gold, P. W., K. E. Gabry, M. R. Yasuda and G. P. Chrousos (2002). "Divergent endocrine abnormalities in melancholic and atypical depression: clinical and pathophysiologic implications." Endocrinol Metab Clin North Am **31**(1): 37-62, vi.
Gonzalez-Rey, E., A. FeRNAndez-Martin, A. Chorny and M. Delgado (2006). "Therapeutic effect of urocortin and adrenomedullin in a murine model of Crohn's disease." Gut **55**(6): 824-832.
Gonzalez-Robayna, I. J., A. E. Falender, S. Ochsner, G. L. Firestone and J. S. Richards (2000). "Follicle-Stimulating Hormone (FSH) Stimulates Phosphorylation and Activation of Protein Kinase B (PKB/AKT) and Serum and Glucocorticoid-Induced Kinase (Sgk): Evidence for A Kinase-Independent Signaling by FSH in Granulosa Cells." Molecular Endocrinology **14**(8): 1283-1300.
Gonzalez, G. A. and M. R. Montminy (1989). "Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133." Cell **59**(4): 675-680.
Gospodarowicz, D., N. Ferrara, L. Schweigerer and G. Neufeld (1987). "Structural characterization and biological functions of fibroblast growth factor." Endocr Rev **8**(2): 95-114.
Graham, C. H., T. S. Hawley, R. G. Hawley, J. R. MacDougall, R. S. Kerbel, N. Khoo and P. K. Lala (1993). "Establishment and characterization of first trimester human trophoblast cells with extended lifespan." Exp Cell Res **206**(2): 204-211.
Graham, C. H., J. J. Lysiak, K. R. McCrae and P. K. Lala (1992). "Localization of transforming growth factor-beta at the human fetal-maternalinterface: role in trophoblast growth and differentiation." Biol Reprod **46**(4): 561-572.
Grammatopoulos, D., G. M. Stirrat, S. A. Williams and E. W. Hillhouse (1996). "The biological activity of the corticotropin-releasing hormone receptor-adenylate cyclase complex

in human myometrium is reduced at the end of pregnancy." J Clin Endocrinol Metab **81**(2): 745-751.
Grammatopoulos, D. K. (2007). "The role of CRH receptors and their agonists in myometrial contractility and quiescence during pregnancy and labour." Front Biosci **12**: 561-571.
Grammatopoulos, D. K. (2008). "Placental corticotrophin-releasing hormone and its receptors in human pregnancy and labour: still a scientific enigma." J Neuroendocrinol **20**(4): 432-438.
Grammatopoulos, D. K. (2008). "Placental Corticotrophin-Releasing Hormone and its Receptors in Human Pregnancy and Labour: Still a Scientific Enigma." JouRNAl of Neuroendocrinology **20**(4): 432-438.
Grammatopoulos, D. K. (2012). "Insights into mechanisms of corticotropin-releasing hormone receptor signal transduction." Br J Pharmacol **166**(1): 85-97.
Grammatopoulos, D. K. and G. P. Chrousos (2002). "Functional characteristics of CRH receptors and potential clinical applications of CRH-receptor antagonists." Trends Endocrinol Metab **13**(10): 436-444.
Grammatopoulos, D. K., Y. Dai, H. S. Randeva, M. A. Levine, E. Karteris, A. J. Easton and E. W. Hillhouse (1999). "A novel spliced variant of the type 1 corticotropin-releasing hormone receptor with a deletion in the seventh transmembrane domain present in the human pregnant term myometrium and fetal membranes." Mol Endocrinol **13**(12): 2189-2202.
Grammatopoulos, D. K. and E. W. Hillhouse (1999). "Basal and interleukin-1beta-stimulated prostaglandin production from cultured human myometrial cells: differential regulation by corticotropin-releasing hormone." J Clin Endocrinol Metab **84**(6): 2204-2211.
Grammatopoulos, D. K., H. S. Randeva, M. A. Levine, K. A. Kanellopoulou and E. W. Hillhouse (2001). "Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins." J Neurochem **76**(2): 509-519.
Greijer, A. E. and E. van der Wall (2004). "The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis." JouRNAl of Clinical Pathology **57**(10): 1009-1014.
Griesinger, G., L. Saleh, S. Bauer, P. Husslein and M. Knofler (2001). "Production of pro- and anti-inflammatory cytokines of human placental trophoblasts in response to pathogenic bacteria." J Soc Gynecol Investig **8**(6): 334-340.
Grigoriadis, D. E., T. W. Lovenberg, D. T. Chalmers, C. Liaw and E. B. De Souze (1996). "Characterization of corticotropin-releasing factor receptor subtypes." Ann N Y Acad Sci **780**: 60-80.
Grino, M., G. P. Chrousos and A. N. Margioris (1987). "The corticotropin releasing hormone gene is expressed in human placenta." Biochem Biophys Res Commun **148**(3): 1208-1214.
Grocott, M. P., D. S. Martin, D. Z. Levett, R. McMorrow, J. Windsor and H. E. Montgomery (2009). "Arterial blood gases and oxygen content in climbers on Mount Everest." N Engl J Med **360**(2): 140-149.
Gu, Y., D. F. Lewis and Y. Wang (2008). "Placental Productions and Expressions of Soluble Endoglin, Soluble fms-Like Tyrosine Kinase Receptor-1, and Placental Growth Factor in Normal and Preeclamptic Pregnancies." The JouRNAl of Clinical Endocrinology & Metabolism **93**(1): 260-266.
Guleria, I. and J. W. Pollard (2000). "The trophoblast is a component of the innate immune system during pregnancy." Nat Med **6**(5): 589-593.
Haeger, P., M. E. Andres, M. I. Forray, C. Daza, S. Araneda and K. Gysling (2006). "Estrogen receptors alpha and beta differentially regulate the transcriptional activity of the Urocortin gene." J Neurosci **26**(18): 4908-4916.
Hai, T. and M. G. Hartman (2001). "The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis." Gene **273**(1): 1-11.

Hammer, F. and P. M. Stewart (2006). "Cortisol metabolism in hypertension." Best Pract Res Clin Endocrinol Metab **20**(3): 337-353.
Harville, E. W., D. A. Savitz, N. Dole, A. H. Herring, J. M. Thorp and K. C. Light (2008). "Stress and placental resistance measured by Doppler ultrasound in early and mid-pregnancy." Ultrasound Obstet Gynecol **32**(1): 23-30.
Hashimoto, K., M. Nishiyama, Y. Tanaka, T. Noguchi, K. Asaba, P. N. Hossein, T. Nishioka and S. Makino (2004). "Urocortins and corticotropin releasing factor type 2 receptors in the hypothalamus and the cardiovascular system." Peptides **25**(10): 1711-1721.
Hatano, N., Y. Mori, M. Oh-hora, A. Kosugi, T. Fujikawa, N. Nakai, H. Niwa, J. Miyazaki, T. Hamaoka and M. Ogata (2003). "Essential role for ERK2 mitogen-activated protein kinase in placental development." Genes Cells **8**(11): 847-856.
Hatzoglou, A., A. N. Margioris, E. Bakogeorgou, A. Gravanis and E. Castanas (1996). "Identification, characterization and localization of corticotropin-releasing hormone receptors in human placenta." Life Sci **59**(22): 1871-1879.
He, Y., S. K. Smith, K. A. Day, D. E. Clark, D. R. Licence and D. S. Charnock-Jones (1999). "AlteRNAtive splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity." Mol Endocrinol **13**(4): 537-545.
Herse, F. and B. LaMarca (2013). "Angiotensin II type 1 receptor autoantibody (AT1-AA)-mediated pregnancy hypertension." Am J Reprod Immunol **69**(4): 413-418.
Heydarian, M., T. McCaffrey, L. Florea, Z. Yang, M. M. Ross, W. Zhou and S. E. Maynard (2009). "Novel splice variants of sFLT-1 are upregulated in preeclampsia." Placenta **30**(3): 250-255.
Hillhouse, E. W. and D. K. Grammatopoulos (2001). "Control of intracellular signalling by corticotropin-releasing hormone in human myometrium." Front Horm Res **27**: 66-74.
Hillhouse, E. W. and D. K. Grammatopoulos (2002). "Role of stress peptides during human pregnancy and labour." Reproduction **124**(3): 323-329.
Hillhouse, E. W. and D. K. Grammatopoulos (2006). "The molecular mechanisms underlying the regulation of the biological activity of corticotropin-releasing hormone receptors: implications for physiology and pathophysiology." Endocr Rev **27**(3): 260-286.
Hiratsuka, S., O. Minowa, J. Kuno, T. Noda and M. Shibuya (1998). "FLT-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice." Proc Natl Acad Sci U S A **95**(16): 9349-9354.
Hisamoto, K., M. Ohmichi, Y. Kanda, K. Adachi, Y. Nishio, J. Hayakawa, S. Mabuchi, K. Takahashi, K. Tasaka, Y. Miyamoto, K. Hisamoto, M. Ohmichi, Y. Kanda, K. Adachi, Y. Nishio, J. Hayakawa, S. Mabuchi, K. Takahashi, K. Tasaka and Y. Miyamoto (2001). "Induction of endothelial nitric-oxide synthase phosphorylation by the raloxifene analog LY117018 is differentially mediated by AKT and extracellular signal-regulated protein kinase in vascular endothelial cells." JouRNAl of Biological Chemistry **276**: 47642-47649.
Hisamoto, K., M. Ohmichi, H. Kurachi, J. Hayakawa, Y. Kanda, Y. Nishio, K. Adachi, K. Tasaka, E. Miyoshi, N. Fujiwara, K. Hisamoto, M. Ohmichi, H. Kurachi, J. Hayakawa, Y. Kanda, Y. Nishio, K. Adachi, K. Tasaka, E. Miyoshi and N. Fujiwara (2001). "Estrogen induces the AKT-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells." JouRNAl of Biological Chemistry **276**: 3459-3467.
Hoeller, A., L. Ehrlich, M. Golic, F. Herse, F. H. Perschel, M. Siwetz, W. Henrich, R. Dechend, B. Huppertz and S. Verlohren (2017). "Placental expression of sFLT-1 and PLGF in early preeclampsia vs. early IUGR vs. age-matched healthy pregnancies." Hypertens Pregnancy **36**(2): 151-160.
Hogg, K., J. D. Blair, D. E. McFadden, P. von Dadelszen and W. P. Robinson (2013). "Early onset pre-eclampsia is associated with altered DNA methylation of cortisol-signalling and steroidogenic genes in the placenta." PLoS One **8**(5): e62969.

Holder, B., C. Tower, V. Abrahams, J. Aplin, B. Holder, C. Tower, V. Abrahams and J. Aplin (2012). "Syncytin 1 in the human placenta." Placenta **33**: 460-466.
Holmes, M. C., C. T. Abrahamsen, K. L. French, J. M. Paterson, J. J. Mullins and J. R. Seckl (2006). "The mother or the fetus? 11beta-hydroxysteroid dehydrogenase type 2 null mice provide evidence for direct fetal programming of behavior by endogenous glucocorticoids." J Neurosci **26**(14): 3840-3844.
Holmlund, U., G. Cebers, A. R. Dahlfors, B. Sandstedt, K. Bremme, E. S. Ekstrom and A. Scheynius (2002). "Expression and regulation of the pattern recognition receptors Toll-like receptor-2 and Toll-like receptor-4 in the human placenta." Immunology **107**(1): 145-151.
Horlick, R. A., K. Sperle, L. A. Breth, C. C. Reid, E. S. Shen, A. K. Robbins, G. M. Cooke and B. L. Largent (1997). "Rapid generation of stable cell lines expressing corticotropin-releasing hormone receptor for drug discovery." Protein Expr Purif **9**(3): 301-308.
Horne, A. W., J. L. V. Shaw, A. Murdoch, S. E. McDonald, A. R. Williams, H. N. Jabbour, W. C. Duncan and H. O. D. Critchley (2011). "Placental growth factor: a promising diagnostic biomarker for tubal ectopic pregnancy." The JouRNAl of clinical endocrinology and metabolism **96**(1): E104-E108.
Hornig, C., B. Barleon, S. Ahmad, P. Vuorela, A. Ahmed and H. A. Weich (2000). "Release and complex formation of soluble VEGFR-1 from endothelial cells and biological fluids." Lab Invest **80**(4): 443-454.
Hsu, S. Y. and A. J. Hsueh (2001). "Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor." Nat Med **7**(5): 605-611.
Hsu, T. Y., K. C. Lan, C. C. Tsai, C. Y. Ou, B. H. Cheng, M. Y. Tsai, H. Y. Kang, Y. H. Tung, Y. H. Wong and K. E. Huang (2009). "Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies." Taiwan J Obstet Gynecol **48**(3): 262-267.
Hu, Z., P. J. Killion and V. R. Iyer (2007). "Genetic reconstruction of a functional transcriptional regulatory network." Nat Genet **39**(5): 683-687.
Huang, Q., H. Chen, J. Li, M. Oliver, X. Ma, D. Byck, Y. Gao, S. Jiang, Q. Huang, H. Chen, J. Li, M. Oliver, X. Ma, D. Byck, Y. Gao and S. Jiang (2014). "Epigenetic and non-epigenetic regulation of syncytin-1 expression in human placenta and cancer tissues." Cellular Signalling **26**: 648-656.
Huang, Q., H. Chen, F. Wang, B. Brost, J. Li, Y. Gao, Z. Li, S. Jiang, Q. Huang, H. Chen, F. Wang, B. Brost, J. Li, Y. Gao, Z. Li and S. Jiang (2014). "Reduced syncytin-1 expression in choriocarcinoma BEWO cells activates the calpain1-AIF-mediated apoptosis, implication for preeclampsia." Cellular and Molecular Life Sciences **71**: 3151-3164.
Huckle, W. R. (2017). Molecular biology and translational science.
Hung, T., J. Skepper, G. Burton, T. Hung, J. Skepper and G. Burton (2001). "In vitro ischemia-reperfusion injury in term human placenta as a model for oxidative stress in pathological pregnancies." American JouRNAl of Pathology **159**: 1031-1043.
Huppertz, B., G. Burton, J. C. Cross and J. C. Kingdom (2006). "Placental morphology: from molecule to mother -- a dedication to Peter Kaufmann -- a review." Placenta **27 Suppl A**: S3-8.
Huppertz, B., H. G. Frank, J. C. Kingdom, F. Reister and P. Kaufmann (1998). "Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta." Histochem Cell Biol **110**(5): 495-508.
Ignarro, L. J., G. M. Buga, K. S. Wood, R. E. Byrns and G. Chaudhuri (1987). "Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide." Proc Natl Acad Sci U S A **84**(24): 9265-9269.

Ikeda, K., K. Tojo, S. Sato, T. Ebisawa, G. Tokudome, T. Hosoya, M. Harada, O. Nakagawa and K. Nakao (1998). "Urocortin, a newly identified corticotropin-releasing factor-related mammalian peptide, stimulates atrial natriuretic peptide and brain natriuretic peptide secretions from neonatal rat cardiomyocytes." Biochem Biophys Res Commun **250**(2): 298-304.
Im, E., S. H. Rhee, Y. S. Park, C. Fiocchi, Y. Tache and C. Pothoulakis (2010). "Corticotropin-releasing hormone family of peptides regulates intestinal angiogenesis." Gastroenterology **138**(7): 2457-2467, 2467.e2451-2455.
Im, E., S. H. Rhee, Y. S. Park, C. Fiocchi, Y. Taché and C. Pothoulakis (2010). "Corticotropin-Releasing Hormone Family of Peptides Regulates Intestinal Angiogenesis." Gastroenterology **138**(7): 2457-2467.e2455.
Im, E., A. Venkatakrishnan and A. Kazlauskas (2005). "Cathepsin B Regulates the Intrinsic Angiogenic Threshold of Endothelial Cells." Molecular Biology of the Cell **16**(8): 3488-3500.
Imperatore, A., P. Florio, P. B. Torres, M. Torricelli, L. Galleri, P. Toti, R. Occhini, E. Picciolini, W. Vale and F. Petraglia (2006). "Urocortin 2 and urocortin 3 are expressed by the human placenta, deciduas, and fetal membranes." Am J Obstet Gynecol **195**(1): 288-295.
Imperatore, A., W. Li, F. Petraglia and J. R. Challis (2009). "Urocortin 2 stimulates estradiol secretion from cultured human placental cells: an effect mediated by the type 2 corticotrophin-releasing hormone (CRH) receptor." Reprod Sci **16**(6): 551-558.
Inada, A., Y. Hamamoto, Y. Tsuura, J.-i. Miyazaki, S. Toyokuni, Y. Ihara, K. Nagai, Y. Yamada, S. Bonner-Weir and Y. Seino (2004). "Overexpression of Inducible Cyclic AMP Early Repressor Inhibits Transactivation of Genes and Cell Proliferation in Pancreatic β Cells." Molecular and Cellular Biology **24**(7): 2831-2841.
Inder, W. J., T. C. Prickett, M. J. Ellis, L. Hull, R. Reid, P. S. Benny, J. H. Livesey and R. A. Donald (2001). "The utility of plasma CRH as a predictor of preterm delivery." J Clin Endocrinol Metab **86**(12): 5706-5710.
Irving, J. A. and P. K. Lala (1995). "Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-beta, IGF-II, and IGFBP-1." Exp Cell Res **217**(2): 419-427.
Isaacs, J. S., Y.-J. Jung, E. G. Mimnaugh, A. Martinez, F. Cuttitta and L. M. Neckers (2002). "Hsp90 Regulates a von Hippel Lindau-independent Hypoxia-inducible Factor-1α-degradative Pathway." JouRNAl of Biological Chemistry **277**(33): 29936-29944.
Ishida, M., T. Mitsui, K. Yamakawa, N. Sugiyama, W. Takahashi, H. Shimura, T. Endo, T. Kobayashi and J. Arita (2007). "Involvement of cAMP response element-binding protein in the regulation of cell proliferation and the prolactin promoter of lactotrophs in primary culture." Am J Physiol Endocrinol Metab **293**(6): E1529-1537.
Ishihara, N., H. Matsuo, H. Murakoshi, J. Laoag-FeRNAndez, T. Samoto, T. Maruo, N.
Ishihara, H. Matsuo, H. Murakoshi, J. Laoag-FeRNAndez, T. Samoto and T. Maruo (2002). "Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation." American JouRNAl of Obstetrics and Gynecology **186**: 158-166.
Ishihara, N., H. Matsuo, H. Murakoshi, J. B. Laoag-FeRNAndez, T. Samoto and T. Maruo (2002). "Increased apoptosis in the syncytiotrophoblast in human term placentas complicated by either preeclampsia or intrauterine growth retardation." Am J Obstet Gynecol **186**(1): 158-166.
Jackson, J. R., M. P. Seed, C. H. Kircher, D. A. Willoughby and J. D. Winkler (1997). "The codependence of angiogenesis and chronic inflammation." The FASEB JouRNAl **11**(6): 457-465.

Jacobs, M., N. Nassar, C. L. Roberts, R. Hadfield, J. M. Morris and A. W. Ashton (2011). "Levels of soluble fms-like tyrosine kinase one in first trimester and outcomes of pregnancy: a systematic review." Reprod Biol Endocrinol **9**: 77.
Jaffe, R. and J. R. Woods, Jr. (1993). "Color Doppler imaging and in vivo assessment of the anatomy and physiology of the early uteroplacental circulation." Fertil Steril **60**(2): 293-297.
Jain, V., S. Q. Shi, Y. P. Vedernikov, G. R. Saade, K. Chwalisz and R. E. Garfield (1998). "In vivo effects of corticotropin-releasing factor in pregnant rats." Am J Obstet Gynecol **178**(1 Pt 1): 186-191.
Jain, V., Y. P. Vedernikov, G. R. Saade, K. Chwalisz and R. E. Garfield (1999). "Endothelium-dependent and-independent mechanisms of vasorelaxation by corticotropin-releasing factor in pregnant rat uterine artery." JouRNAl of Pharmacology and Experimental Therapeutics **288**(2): 407-413.
Jain, V., Y. P. Vedernikov, G. R. Saade, K. Chwalisz and R. E. Garfield (1999). "Endothelium-dependent and -independent mechanisms of vasorelaxation by corticotropin-releasing factor in pregnant rat uterine artery." J Pharmacol Exp Ther **288**(2): 407-413.
Jansson, N., J. Pettersson, A. Haafiz, A. Ericsson, I. Palmberg, M. Tranberg, V. Ganapathy, T. L. Powell and T. Jansson (2006). "Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in rats fed a low protein diet." The JouRNAl of Physiology **576**(Pt 3): 935-946.
Jauniaux, E., G. Burton, G. Moscosco, J. Hustin, E. Jauniaux, G. Burton, G. Moscosco and J. Hustin (1991). "Development of the early placenta: a morphometric study." Placenta **12**: 269-276.
Jauniaux, E., J. Hempstock, N. Greenwold and G. J. Burton (2003). "Trophoblastic Oxidative Stress in Relation to Temporal and Regional Differences in MaternalPlacental Blood Flow in Normal and Abnormal Early Pregnancies." The American JouRNAl of Pathology **162**(1): 115-125.
Jauniaux, E., D. Jurkovic and S. CAMPbell (1991). "In vivo investigations of the anatomy and the physiology of early human placental circulations." Ultrasound Obstet Gynecol **1**(6): 435-445.
Jauniaux, E., A. Watson, O. Ozturk, D. Quick and G. Burton (1999). "In-vivo measurement of intrauterine gases and acid-base values early in human pregnancy." Hum Reprod **14**(11): 2901-2904.
Jauniaux, E., A. L. Watson, J. Hempstock, Y. P. Bao, J. N. Skepper and G. J. Burton (2000). "Onset of maternalarterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure." Am J Pathol **157**(6): 2111-2122.
Jelkmann, W. (2001). "Pitfalls in the measurement of circulating vascular endothelial growth factor." Clin Chem **47**(4): 617-623.
Jeon, S. H., B. C. Chae, H. A. Kim, G. Y. Seo, D. W. Seo, G. T. Chun, S. W. Yie, S. H. Eom and P. H. Kim (2007). "The PKA/CREB pathway is closely involved in VEGF expression in mouse macrophages." Mol Cells **23**(1): 23-29.
Jeschke, M. G., R. P. Mlcak, C. C. Finnerty, W. B. Norbury, G. G. Gauglitz, G. A. Kulp and D. N. Herndon (2007). "Burn size determines the inflammatory and hypermetabolic response." Critical Care **11**(4): R90.
Jhala, U. S., G. Canettieri, R. A. Screaton, R. N. Kulkarni, S. Krajewski, J. Reed, J. Walker, X. Lin, M. White and M. Montminy (2003). "cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2." Genes Dev **17**(13): 1575-1580.
Jiang, B., A. Kamat and C. R. Mendelson (2000). "Hypoxia Prevents Induction of Aromatase Expression in Human Trophoblast Cells in Culture: Potential Inhibitory Role of the Hypoxia-Inducible Transcription Factor Mash-2 (Mammalian Achaete-Scute Homologous Protein-2)." Molecular Endocrinology **14**(10): 1661-1673.

Jirkovska, M., J. Janacek, J. Kalab, L. Kubinova, M. Jirkovska, J. Janacek, J. Kalab and L. Kubinova (2008). "Three-dimensional arrangement of the capillary bed and its relationship to microrheology in the terminal villi of normal term placenta." Placenta **29**: 892-897.
Johnstone, E. D., C. P. Sibley, B. Lowen and L. J. Guilbert (2005). "Epidermal growth factor stimulation of trophoblast differentiation requires MAPK11/14 (p38 MAP kinase) activation." Biol Reprod **73**(6): 1282-1288.
Jolley, S. N., S. Elmore, K. E. BaRNArd and D. B. Carr (2007). "Dysregulation of the hypothalamic-pituitary-adrenal axis in postpartum depression." Biol Res Nurs **8**(3): 210-222.
Jones, S. A., A. N. Brooks and J. R. Challis (1989). "Steroids modulate corticotropin-releasing hormone production in human fetal membranes and placenta." J Clin Endocrinol Metab **68**(4): 825-830.
Jones, S. A. and J. R. Challis (1989). "Local stimulation of prostaglandin production by corticotropin-releasing hormone in human fetal membranes and placenta." Biochem Biophys Res Commun **159**(1): 192-199.
Jones, S. A. and J. R. Challis (1990). "Effects of corticotropin-releasing hormone and adrenocorticotropin on prostaglandin output by human placenta and fetal membranes." Gynecol Obstet Invest **29**(3): 165-168.
Kalantaridou, S. N., A. Makrigiannakis, G. Mastorakos and G. P. Chrousos (2003). "Roles of reproductive corticotropin-releasing hormone." Ann N Y Acad Sci **997**: 129-135.
Kam, E. P. Y., L. Gardner, Y. W. Loke and A. King (1999). "The role of trophoblast in the physiological change in decidual spiral arteries." Human Reproduction **14**(8): 2131-2138.
Kang, M. C., S. J. Park, H. J. Kim, J. Lee, D. H. Yu, K. B. Bae, Y. R. Ji, S. J. Park, J. Jeong, W. Y. Jang, J. H. Kim, M. S. Choi, D. S. Lee, H. S. Lee, S. Lee, S. H. Kim, M. O. Kim, G. Park, Y. S. Choo, J. Y. Cho and Z. Y. Ryoo (2014). "Gestational loss and growth restriction by angiogenic defects in placental growth factor transgenic mice." Arterioscler Thromb Vasc Biol **34**(10): 2276-2282.
Karalis, K., G. Goodwin and J. A. Majzoub (1996). "Cortisol blockade of progesterone: a possible molecular mechanism involved in the initiation of human labor." Nat Med **2**(5): 556-560.
Karalis, K., H. Sano, J. Redwine, S. Listwak, R. L. Wilder and G. P. Chrousos (1991). "Autocrine or paracrine inflammatory actions of corticotropin-releasing hormone in vivo." Science **254**(5030): 421-423.
Karimu, A., G. Burton, A. Karimu and G. Burton (1994). "The effects of maternalvascular pressure on the dimensions of the placental capillaries." British JouRNAl of Obstetrics and Gynaecology **101**: 57-63.
Karimu, A. L. and G. J. Burton (1994). "The effects of maternalvascular pressure on the dimensions of the placental capillaries." Br J Obstet Gynaecol **101**(1): 57-63.
Karteris, E., A. Goumenou, E. Koumantakis, E. W. Hillhouse and D. K. Grammatopoulos (2003). "Reduced expression of corticotropin-releasing hormone receptor type-1 alpha in human preeclamptic and growth-restricted placentas." J Clin Endocrinol Metab **88**(1): 363-370.
Karteris, E., A. Goumenou, E. Koumantakis, E. W. Hillhouse and D. K. Grammatopoulos (2003). "Reduced Expression of Corticotropin-Releasing Hormone Receptor Type-1α in Human Preeclamptic and Growth-Restricted Placentas." The JouRNAl of Clinical Endocrinology & Metabolism **88**(1): 363-370.
Karteris, E., D. Grammatopoulos, H. Randeva and E. W. Hillhouse (2000). "Signal transduction characteristics of the corticotropin-releasing hormone receptors in the feto-placental unit." J Clin Endocrinol Metab **85**(5): 1989-1996.

Karteris, E., D. K. Grammatopoulos, H. S. Randeva and E. W. Hillhouse (2001). "The role of corticotropin-releasing hormone receptors in placenta and fetal membranes during human pregnancy." Mol Genet Metab **72**(4): 287-296.
Karteris, E., E. W. Hillhouse and D. Grammatopoulos (2004). "Urocortin II is expressed in human pregnant myometrial cells and regulates myosin light chain phosphorylation: potential role of the type-2 corticotropin-releasing hormone receptor in the control of myometrial contractility." Endocrinology **145**(2): 890-900.
Karteris, E., M. Vatish, E. W. Hillhouse and D. K. Grammatopoulos (2005). "Preeclampsia is associated with impaired regulation of the placental nitric oxide-cyclic guanosine monophosphate pathway by corticotropin-releasing hormone (CRH) and CRH-related peptides." J Clin Endocrinol Metab **90**(6): 3680-3687.
Karumanchi, S. A. and Y. Bdolah (2004). "Hypoxia and sFLT-1 in preeclampsia: the "chicken-and-egg" question." Endocrinology **145**(11): 4835-4837.
Karumanchi, S. A. and I. E. Stillman (2006). "In vivo rat model of preeclampsia." Methods Mol Med **122**: 393-399.
Kaufmann, P., T. Mayhew, D. Charnock Jones, P. Kaufmann, T. Mayhew and D. Charnock Jones (2004). "Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy." Placenta **25**: 114-126.
Kaufmann, P., T. M. Mayhew and D. S. Charnock-Jones (2004). "Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy." Placenta **25**(2-3): 114-126.
Kauma, S. W., V. Bae-Jump and S. W. Walsh (1999). "Hepatocyte growth factor stimulates trophoblast invasion: a potential mechanism for abnormal placentation in preeclampsia." J Clin Endocrinol Metab **84**(11): 4092-4096.
Keck, P. J., S. D. Hauser, G. Krivi, K. Sanzo, T. Warren, J. Feder and D. T. Connolly (1989). "Vascular permeability factor, an endothelial cell mitogen related to PDGF." Science **246**(4935): 1309-1312.
Keelan, J. A. and M. D. Mitchell (2005). "Cytokines, Hypoxia, and Preeclampsia." JouRNAl of the Society for Gynecologic Investigation **12**(6): 385-387.
Keller, P. A., K. Kirkwood, J. Morgan, S. Westcott and A. McCluskey (2003). "The prevention of preterm labour -- corticotropin releasing hormone type 1 receptors as a target for drug design and development." Mini Rev Med Chem **3**(4): 295-303.
Kendall, R. L. and K. A. Thomas (1993). "Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor." Proceedings of the National Academy of Sciences **90**(22): 10705-10709.
Kendall, R. L. and K. A. Thomas (1993). "Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor." Proc Natl Acad Sci U S A **90**(22): 10705-10709.
Kendall, R. L. and K. A. Thomas (1993). "Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor." Proceedings of the National Academy of Sciences of the United States of America **90**(22): 10705-10709.
Kendall, R. L., G. Wang and K. A. Thomas (1996). "Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR." Biochem Biophys Res Commun **226**(2): 324-328.
Kent, L., T. Konno and M. Soares (2010). "Phosphatidylinositol 3 kinase modulation of trophoblast cell differentiation." BMC Developmental Biology **10**(1): 97.
Keryer, G., E. Alsat, K. Tasken and D. Evain-Brion (1998). "Cyclic AMP-dependent protein kinases and human trophoblast cell differentiation in vitro." J Cell Sci **111 (Pt 7)**: 995-1004.

Keryer, G., E. Alsat, K. Taskén and D. Evain-Brion (1998). "Role of cyclic AMP-dependent protein kinases in human villous cytotrophoblast differentiation: A review." Placenta **19**: 295-314.
Keyes, L. E., J. F. Armaza, S. Niermeyer, E. Vargas, D. A. Young and L. G. Moore (2003). "Intrauterine growth restriction, preeclampsia, and intrauterine mortality at high altitude in Bolivia." Pediatr Res **54**(1): 20-25.
Khaliq, A., C. Dunk, J. Jiang, M. Shams, X. F. Li, C. Acevedo, H. Weich, M. Whittle and A. Ahmed (1999). "Hypoxia down-regulates placenta growth factor, whereas fetal growth restriction up-regulates placenta growth factor expression: molecular evidence for "placental hyperoxia" in intrauterine growth restriction." Lab Invest **79**(2): 151-170.
Kim, D. H., D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst and D. M. Sabatini (2002). "MTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery." Cell **110**(2): 163-175.
Kim, S., K. Jee, D. Kim, H. Koh and J. Chung (2001). "Cyclic AMP Inhibits AKT Activity by Blocking the Membrane Localization of PDK1." JouRNAl of Biological Chemistry **276**(16): 12864-12870.
Kimura, Y., K. Takahashi, K. Totsune, Y. Muramatsu, C. Kaneko, A. D. Darnel, T. Suzuki, M. Ebina, T. Nukiwa and H. Sasano (2002). "Expression of urocortin and corticotropin-releasing factor receptor subtypes in the human heart." J Clin Endocrinol Metab **87**(1): 340-346.
Kingdom, J., P. Kaufmann, J. Kingdom and P. Kaufmann (1997). "Oxygen and placental villous development: origins of fetal hypoxia." Placenta **18**: 613-621.
Kingdom, J. C. and P. Kaufmann (1997). "Oxygen and placental villous development: origins of fetal hypoxia." Placenta **18**(8): 613-621; discussion 623-616.
Kleinrouweler, C. E., M. M. Wiegerinck, C. Ris-Stalpers, P. M. Bossuyt, J. A. van der Post, P. von Dadelszen, B. W. Mol and E. Pajkrt (2012). "Accuracy of circulating placental growth factor, vascular endothelial growth factor, soluble fms-like tyrosine kinase 1 and soluble endoglin in the prediction of pre-eclampsia: a systematic review and meta-analysis." Bjog **119**(7): 778-787.
Kliman, H. J., J. E. Nestler, E. Sermasi, J. M. Sanger and J. F. Strauss, 3rd (1986). "Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae." Endocrinology **118**(4): 1567-1582.
Klimaviciute, A., J. Calciolari, E. Bertucci, S. Abelin-Tornblom, Y. Stjernholm-Vladic, B. Bystrom, F. Petraglia and G. Ekman-Ordeberg (2006). "Corticotropin-releasing hormone, its binding protein and receptors in human cervical tissue at preterm and term labor in comparison to non-pregnant state." Reprod Biol Endocrinol **4**: 29.
Knerr, I., E. Beinder and W. Rascher (2002). "Syncytin, a novel human endogenous retroviral gene in human placenta: evidence for its dysregulation in preeclampsia and HELLP syndrome." Am J Obstet Gynecol **186**(2): 210-213.
Knerr, I., M. Schnare, K. Hermann, S. Kausler, M. Lehner, T. Vogler, W. Rascher, U. Meissner, I. Knerr, M. Schnare, K. Hermann, S. Kausler, M. Lehner, T. Vogler, W. Rascher and U. Meissner (2007). "Fusiogenic endogenous-retroviral syncytin-1 exerts anti-apoptotic functions in staurosporine-challenged CHO cells." Apoptosis **12**: 37-43.
Knerr, I., S. W. Schubert, C. Wich, K. Amann, T. Aigner, T. Vogler, R. Jung, J. Dotsch, W. Rascher and S. Hashemolhosseini (2005). "Stimulation of GCMa and syncytin via cAMP mediated PKA signaling in human trophoblastic cells under normoxic and hypoxic conditions." FEBS Lett **579**(18): 3991-3998.
Knerr, I., S. W. Schubert, C. Wich, K. Amann, T. Aigner, T. Vogler, R. Jung, J. Dötsch, W. Rascher and S. Hashemolhosseini (2005). "Stimulation of GCMa and syncytin via cAMP

mediated PKA signaling in human trophoblastic cells under normoxic and hypoxic conditions." FEBS Letters **579**(18): 3991-3998.
Knerr, I., S. Soder, E. Licha, T. Aigner, W. Rascher, I. Knerr, S. Soder, E. Licha, T. Aigner and W. Rascher (2008). "Response of HEK293 and CHO cells overexpressing fusiogenic syncytin-1 to mitochondrion-mediated apoptosis induced by antimycin A." JouRNAl of Cellular Biochemistry **105**: 766-775.
Knofler, M., L. Saleh, H. Strohmer, P. Husslein and M. F. Wolschek (1999). "Cyclic AMP- and differentiation-dependent regulation of the proximal alphaHCG gene promoter in term villous trophoblasts." Mol Hum Reprod **5**(6): 573-580.
Knuth, A., L. Liu, H. Nielsen, D. Merril, D. S. Torry and J. A. Arroyo (2015). "Placenta growth factor induces invasion and activates p70 during rapamycin treatment in trophoblast cells." Am J Reprod Immunol **73**(4): 330-340.
Knuth, N. D., D. L. Johannsen, R. A. Tamboli, P. A. Marks-Shulman, R. Huizenga, K. Y. Chen, N. N. Abumrad, E. Ravussin and K. D. Hall (2014). "Metabolic adaptation following massive weight loss is related to the degree of energy imbalance and changes in circulating leptin." Obesity (Silver Spring) **22**(12): 2563-2569.
Koga, K., Y. Osuga, O. Yoshino, Y. Hirota, X. Ruimeng, T. Hirata, S. Takeda, T. Yano, O. Tsutsumi and Y. Taketani (2003). "Elevated serum soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) levels in women with preeclampsia." J Clin Endocrinol Metab **88**(5): 2348-2351.
Koga, K., Y. Osuga, O. Yoshino, Y. Hirota, X. Ruimeng, T. Hirata, S. Takeda, T. Yano, O. Tsutsumi and Y. Taketani (2003). "Elevated Serum Soluble Vascular Endothelial Growth Factor Receptor 1 (sVEGFR-1) Levels in Women with Preeclampsia." The JouRNAl of Clinical Endocrinology & Metabolism **88**(5): 2348-2351.
Kokkotou, E., D. Torres, A. C. Moss, M. O'Brien, D. E. Grigoriadis, K. Karalis and C. Pothoulakis (2006). "Corticotropin-Releasing Hormone Receptor 2-Deficient Mice Have Reduced Intestinal Inflammatory Responses." The JouRNAl of Immunology **177**(5): 3355-3361.
Kondo, K., S. Hiratsuka, E. Subbalakshmi, H. Matsushime and M. Shibuya (1998). "Genomic organization of the FLT-1 gene encoding for vascular endothelial growth factor (VEGF) receptor-1 suggests an intimate evolutionary relationship between the 7-Ig and the 5-Ig tyrosine kinase receptors." Gene **208**(2): 297-305.
Korebrits, C., M. M. Ramirez, L. Watson, E. Brinkman, A. D. Bocking and J. R. Challis (1998). "Maternalcorticotropin-releasing hormone is increased with impending preterm birth." J Clin Endocrinol Metab **83**(5): 1585-1591.
Kostich, W. A., A. Chen, K. Sperle and B. L. Largent (1998). "Molecular identification and analysis of a novel human corticotropin-releasing factor (CRF) receptor: the CRF2gamma receptor." Mol Endocrinol **12**(8): 1077-1085.
Kotz, C. M., C. Wang, A. S. Levine and C. J. Billington (2002). "Urocortin in the hypothalamic PVN increases leptin and affects uncoupling proteins-1 and -3 in rats." Am J Physiol Regul Integr Comp Physiol **282**(2): R546-551.
Kovalovsky, D. n., D. n. Refojo, A. C. Liberman, D. Hochbaum, M. P. Pereda, O. A. Coso, G. n. K. Stalla, F. Holsboer and E. Arzt (2002). "Activation and Induction of NUR77/NURR1 in Corticotrophs by CRH/cAMP: Involvement of Calcium, Protein Kinase A, and MAPK Pathways." Molecular Endocrinology **16**(7): 1638-1651.
Kraiem, Z., O. Sadeh, D. L. Blithe and B. C. Nisula (1994). "Human chorionic gonadotropin stimulates thyroid hormone secretion, iodide uptake, organification, and adenosine 3',5'-monophosphate formation in cultured human thyrocytes." J Clin Endocrinol Metab **79**(2): 595-599.

Krebs, C., L. M. Macara, R. Leiser, A. W. Bowman, I. A. Greer and J. C. Kingdom (1996). "Intrauterine growth restriction with absent end-diastolic flow velocity in the umbilical artery is associated with maldevelopment of the placental terminal villous tree." Am J Obstet Gynecol **175**(6): 1534-1542.
Krysiak, O., A. Bretschneider, E. Zhong, J. Webb, H. Hopp, S. Verlohren, N. Fuhr, M. Lanowska, A. Nonnenmacher, R. Vetter, J. Jankowski, M. Paul and G. Schonfelder (2005). "Soluble vascular endothelial growth factor receptor-1 (sFLT-1) mediates downregulation of FLT-1 and prevents activated neutrophils from women with preeclampsia from additional migration by VEGF." Circ Res **97**(12): 1253-1261.
Kudo, Y., C. Boyd, I. Sargent, C. Redman, Y. Kudo, C. Boyd, I. Sargent and C. Redman (2003). "Hypoxia alters expression and function of syncytin and its receptor during trophoblast cell fusion of human placental BEWO cells: implications for impaired trophoblast syncytialisation in pre-eclampsia." Biochimica et Biophysica Acta **1638**: 63-71.
Kudo, Y., C. A. Boyd, I. L. Sargent and C. W. Redman (2003). "Hypoxia alters expression and function of syncytin and its receptor during trophoblast cell fusion of human placental BEWO cells: implications for impaired trophoblast syncytialisation in pre-eclampsia." Biochim Biophys Acta **1638**(1): 63-71.
Kumazaki, K., M. Nakayama, I. Yanagihara, N. Suehara and Y. Wada (2004). "Immunohistochemical distribution of Toll-like receptor 4 in term and preterm human placentas from normal and complicated pregnancy including chorioamnionitis." Hum Pathol **35**(1): 47-54.
Kurtzman, J. T., H. Wilson and C. V. Rao (2001). "A proposed role for hCG in clinical obstetrics." Semin Reprod Med **19**(1): 63-68.
Kuzmina, I., G. Hubina-Vakulik, G. Burton, I. Kuzmina, G. Hubina-Vakulik and G. Burton (2005). "Placental morphometry and Doppler flow velocimetry in cases of chronic human fetal hypoxia." European JouRNAl of Obstetrics, Gynecology, and Reproductive Biology **120**: 139-145.
Land, S. C. and A. R. Tee (2007). "Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (MTOR) via an MTOR signaling motif." J Biol Chem **282**(28): 20534-20543.
Larsson, L., B. Bjerregaard, L. Wulf-Andersen, J. Talts, L. Larsson, B. Bjerregaard, L. Wulf-Andersen and J. Talts (2007). "Syncytin and cancer cell fusions." Scientific World JouRNAl **7**: 1193-1197.
Leach, L. and L. Leach (2002). "The phenotype of the human materno-fetal endothelial barrier: molecular occupancy of paracellular junctions dictate permeability and angiogenic plasticity." JouRNAl of Anatomy **200**: 599-606.
LeCouter, J., J. Kowalski, J. Foster, P. Hass, Z. Zhang, L. Dillard-Telm, G. Frantz, L. Rangell, L. DeGuzman, G. A. Keller, F. Peale, A. Gurney, K. J. Hillan and N. Ferrara (2001). "Identification of an angiogenic mitogen selective for endocrine gland endothelium." Nature **412**(6850): 877-884.
Lee, J. S., D. J. Jang, N. Lee, H. G. Ko, H. Kim, Y. S. Kim, B. Kim, J. Son, S. H. Kim, H. Chung, M. Y. Lee, W. R. Kim, W. Sun, M. Zhuo, T. Abel, B. K. Kaang and H. Son (2009). "Induction of neuronal vascular endothelial growth factor expression by cAMP in the dentate gyrus of the hippocAMPus is required for antidepressant-like behaviors." J Neurosci **29**(26): 8493-8505.
Lee, X., J. C. Keith, Jr., N. Stumm, I. Moutsatsos, J. M. McCoy, C. P. Crum, D. Genest, D. Chin, C. Ehrenfels, R. Pijnenborg, F. A. van Assche and S. Mi (2001). "Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia." Placenta **22**(10): 808-812.

Lee, X., J. J. Keith, N. Stumm, I. Moutsatsos, J. McCoy, C. Crum, D. Genest, D. Chin, C. Ehrenfels, R. Pijnenborg, X. Lee, J. J. Keith, N. Stumm, I. Moutsatsos, J. McCoy, C. Crum, D. Genest, D. Chin, C. Ehrenfels and R. Pijnenborg (2001). "Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia." Placenta **22**: 808-812.
Lei, S., R. Richter, M. Bienert and M. J. Mulvany (1993). "Relaxing actions of corticotropin-releasing factor on rat resistance arteries." Br J Pharmacol **108**(4): 941-947.
Leisser, C., L. Saleh, S. Haider, H. Husslein, S. Sonderegger and M. Knofler (2006). "Tumour necrosis factor-alpha impairs chorionic gonadotrophin beta-subunit expression and cell fusion of human villous cytotrophoblast." Mol Hum Reprod **12**(10): 601-609.
Lennon, C., M. G. Carlson, D. M. Nelson and Y. Sadovsky (1999). "In vitro modulation of the expression of 15-hydroxy-prostaglandin dehydrogenase by trophoblast differentiation." Am J Obstet Gynecol **180**(3 Pt 1): 690-695.
Lenz, H. J., M. Burlage, A. Raedler and H. Greten (1988). "Central nervous system effects of corticotropin-releasing factor on gastrointestinal transit in the rat." Gastroenterology **94**(3): 598-602.
Lenz, H. J., A. Raedler, H. Greten, W. W. Vale and J. E. Rivier (1988). "Stress-Induced Gastrointestinal Secretory and Motor Responses in Rats Are Mediated by Endogenous Corticotropin-Releasing Factor." Gastroenterology **95**(6): 1510-1517.
Leonard, M. O., K. Howell, S. F. Madden, C. M. Costello, D. G. Higgins, C. T. Taylor and P. McLoughlin (2008). "Hypoxia Selectively Activates the CREB Family of Transcription Factors in the In Vivo Lung." American JouRNAl of Respiratory and Critical Care Medicine **178**(9): 977-983.
Leung, D. W., G. Cachianes, W. J. Kuang, D. V. Goeddel and N. Ferrara (1989). "Vascular endothelial growth factor is a secreted angiogenic mitogen." Science **246**(4935): 1306-1309.
Levine, R. J. and S. A. Karumanchi (2005). "Circulating angiogenic factors in preeclampsia." Clin Obstet Gynecol **48**(2): 372-386.
Levine, R. J., S. E. Maynard, C. Qian, K. H. Lim, L. J. England, K. F. Yu, E. F. Schisterman, R. Thadhani, B. P. Sachs, F. H. Epstein, B. M. Sibai, V. P. Sukhatme and S. A. Karumanchi (2004). "Circulating angiogenic factors and the risk of preeclampsia." N Engl J Med **350**(7): 672-683.
Lewis, K., C. Li, M. H. Perrin, A. Blount, K. Kunitake, C. Donaldson, J. Vaughan, T. M. Reyes, J. Gulyas, W. Fischer, L. Bilezikjian, J. Rivier, P. E. Sawchenko and W. W. Vale (2001). "Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor." Proc Natl Acad Sci U S A **98**(13): 7570-7575.
Li, H., J. Dakour, L. Guilbert, B. Winkler-Lowen, F. Lyall, D. Morrish, H. Li, J. Dakour, L. Guilbert, B. Winkler-Lowen, F. Lyall and D. Morrish (2005). "PL74 a novel member of the transforming growth factor-beta superfamily, is overexpressed in preeclampsia and causes apoptosis in trophoblast cells." JouRNAl of Clinical Endocrinology and Metabolism **90**: 3045-3053.
Li, H., B. Gu, Y. Zhang, D. F. Lewis and Y. Wang (2005). "Hypoxia-induced increase in soluble FLT-1 production correlates with enhanced oxidative stress in trophoblast cells from the human placenta." Placenta **26**(2-3): 210-217.
Li, M., X. Wang, M. K. Meintzer, T. Laessig, M. J. Birnbaum and K. A. Heidenreich (2000). "Cyclic AMP Promotes Neuronal Survival by Phosphorylation of Glycogen Synthase Kinase 3β." Molecular and Cellular Biology **20**(24): 9356-9363.
Li, W. and J. R. Challis (2005). "Corticotropin-releasing hormone and urocortin induce secretion of matrix metalloproteinase-9 (MMP-9) without change in tissue inhibitors of

MMP-1 by cultured cells from human placenta and fetal membranes." J Clin Endocrinol Metab **90**(12): 6569-6574.
Liao, W. X., L. Feng, H. Zhang, J. Zheng, T. R. Moore and D. B. Chen (2009). "Compartmentalizing VEGF-induced ERK2/1 signaling in placental artery endothelial cell caveolae: a paradoxical role of caveolin-1 in placental angiogenesis in vitro." Mol Endocrinol **23**(9): 1428-1444.
Liaw, C. W., T. W. Lovenberg, G. Barry, T. Oltersdorf, D. E. Grigoriadis and E. B. de Souza (1996). "Cloning and characterization of the human corticotropin-releasing factor-2 receptor complementary deoxyribonucleic acid." Endocrinology **137**(1): 72-77.
Lin, L., B. Xu and N. S. Rote (1999). "Expression of endogenous retrovirus ERV-3 induces differentiation in BEWO, a choriocarcinoma model of human placental trophoblast." Placenta **20**(1): 109-118.
Linton, E. A., A. V. PERKins, R. J. Woods, F. Eben, C. D. Wolfe, D. P. Behan, E. Potter, W. W. Vale and P. J. Lowry (1993). "Corticotropin releasing hormone-binding protein (CRH-BP): plasma levels decrease during the third trimester of normal human pregnancy." J Clin Endocrinol Metab **76**(1): 260-262.
Lodish, H. F., N. Kong, M. Snider and G. J. A. M. Strous (1983). "Hepatoma secretory proteins migrate from rough endoplasmic reticulum to Golgi at characteristic rates." Nature **304**: 80.
Lovejoy, D. A. and R. J. Balment (1999). "Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates." Gen Comp Endocrinol **115**(1): 1-22.
Lovenberg, T. W., D. T. Chalmers, C. Liu and E. B. De Souza (1995). "CRF2 alpha and CRF2 beta receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues." Endocrinology **136**(9): 4139-4142.
Luft, F. C. (2014). "Soluble fms-like tyrosine kinase-1 and atherosclerosis in chronic kidney disease." Kidney InteRNAtional **85**(2): 238-240.
Luini, A., D. Lewis, S. Guild, D. Corda and J. Axelrod (1985). "Hormone secretagogues increase cytosolic calcium by increasing cAMP in corticotropin-secreting cells." Proceedings of the National Academy of Sciences **82**(23): 8034-8038.
Luna, R. L., V. R. Kay, M. T. Ratsep, K. Khalaj, M. Bidarimath, N. Peterson, P. Carmeliet, A. Jin and B. A. Croy (2016). "Placental growth factor deficiency is associated with impaired cerebral vascular development in mice." Mol Hum Reprod **22**(2): 130-142.
Luo, Z., Y. Fujio, Y. Kureishi, R. Rudic, G. Daumerie, D. Fulton, W. Sessa, K. Walsh, Z. Luo, Y. Fujio, Y. Kureishi, R. Rudic, G. Daumerie, D. Fulton, W. Sessa and K. Walsh (2000). "Acute modulation of endothelial AKT/PKB activity alters nitric oxide-dependent vasomotor activity in vivo." JouRNAl of Clinical Investigation **106**: 493-499.
Luscher, B. P., C. Marini, M. S. Joerger-Messerli, X. Huang, M. A. Hediger, C. Albrecht, M. U. Baumann and D. V. Surbek (2017). "Placental glucose transporter (GLUT)-1 is down-regulated in preeclampsia." Placenta **55**: 94-99.
Luttun, A., M. Autiero, M. Tjwa and P. Carmeliet (2004). "Genetic dissection of tumor angiogenesis: are PLGF and VEGFR-1 novel anti-cancer targets?" Biochim Biophys Acta **1654**(1): 79-94.
Ma, X. M. and J. Blenis (2009). "Molecular mechanisms of MTOR-mediated translational control." Nat Rev Mol Cell Biol **10**(5): 307-318.
Magiakou, M. A., G. Mastorakos, D. Rabin, B. Dubbert, P. W. Gold and G. P. Chrousos (1996). "Hypothalamic corticotropin-releasing hormone suppression during the postpartum period: implications for the increase in psychiatric manifestations at this time." J Clin Endocrinol Metab **81**(5): 1912-1917.

Maglione, D., V. Guerriero, G. Viglietto, P. Delli-Bovi and M. G. Persico (1991). "Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor." Proc Natl Acad Sci U S A **88**(20): 9267-9271.
Majmundar, A. J., W. J. Wong and M. C. Simon (2010). "Hypoxia-inducible factors and the response to hypoxic stress." Mol Cell **40**(2): 294-309.
Major, H. D., R. A. Cambell, R. M. Silver, D. W. Branch and A. S. Weyrich (2014). "Synthesis of sFLT-1 by platelet-monocyte aggregates contributes to the pathogenesis of preeclampsia." American jouRNAl of obstetrics and gynecology **210**(6): 547.e541-547.e547.
Makrigiannakis, A., E. Zoumakis, S. Kalantaridou, N. Mitsiades, A. Margioris, G. P. Chrousos and A. Gravanis (2003). "Corticotropin-releasing hormone (CRH) and immunotolerance of the fetus." Biochem Pharmacol **65**(6): 917-921.
Maldonado-Mercado, M. G., M. T. Espinosa-García, C. Gómez-Concha, J. Monreal-Flores and F. Martinez (2008). "Steroidogenesis in BEWO cells: Role of protein kinase A and benzodiazepines." The InteRNAtional JouRNAl of Biochemistry & Cell Biology **40**(5): 901-908.
Maliniemi, P., M. Vincendeau, J. Mayer, O. Frank, S. Hahtola, L. Karenko, E. Carlsson, F. Mallet, W. Seifarth, C. Leib-Mosch, P. Maliniemi, M. Vincendeau, J. Mayer, O. Frank, S. Hahtola, L. Karenko, E. Carlsson, F. Mallet, W. Seifarth and C. Leib-Mosch (2013). "Expression of human endogenous retrovirus-w including syncytin-1 in cutaneous T-cell lymphoma." PLoS ONE **8**: e76281.
Maltepe, E., A. I. Bakardjiev and S. J. Fisher (2010). "The placenta: transcriptional, epigenetic, and physiological integration during development." J Clin Invest **120**(4): 1016-1025.
Mangeney, M., M. Renard, G. Schlecht-Louf, I. Bouallaga, O. Heidmann, C. Letzelter, A. Richaud, B. Ducos, T. Heidmann, M. Mangeney, M. Renard, G. Schlecht-Louf, I. Bouallaga, O. Heidmann, C. Letzelter, A. Richaud, B. Ducos and T. Heidmann (2007). "Placental syncytins: genetic disjunction between the fusogenic and immunosuppressive activity of retroviral envelope proteins." PNAS **104**: 20534-20539.
Mano, T. and J. Y. Chou (1981). "Regulation of human chorionic gonadotropin synthesis in cultured human placental cells by glucocorticoids." Endocrinology **109**(3): 888-892.
Markovic, D., M. Vatish, M. Gu, D. Slater, R. Newton, H. Lehnert and D. K. Grammatopoulos (2007). "The onset of labor alters corticotropin-releasing hormone type 1 receptor variant expression in human myometrium: putative role of interleukin-1beta." Endocrinology **148**(7): 3205-3213.
Martínez, V., L. Wang, J. E. Rivier, W. Vale and Y. Taché (2002). "Differential Actions of Peripheral Corticotropin-Releasing Factor (CRF), Urocortin II, and Urocortin III on Gastric Emptying and Colonic Transit in Mice: Role of CRF Receptor Subtypes 1 and 2." JouRNAl of Pharmacology and Experimental Therapeutics **301**(2): 611-617.
Maser-Gluth, C., U. Lorenz and P. Vecsei (1987). "In pregnancy, corticotropin-releasing-factor in maternalblood and amniotic fluid correlates with the gestational age." Horm Metab Res Suppl **16**: 42-44.
Mastorakos, G. and I. Ilias (2003). "Maternaland fetal hypothalamic-pituitary-adrenal axes during pregnancy and postpartum." Ann N Y Acad Sci **997**: 136-149.
Mastorakos, G., E. L. Webster, T. C. Friedman and G. P. Chrousos (1993). "Immunoreactive corticotropin-releasing hormone and its binding sites in the rat ovary." J Clin Invest **92**(2): 961-968.
Mata-Greenwood, E., W. X. Liao, W. Wang, J. Zheng and D. B. Chen (2010). "Activation of AP-1 transcription factors differentiates FGF2 and vascular endothelial growth factor regulation of endothelial nitric-oxide synthase expression in placental artery endothelial cells." J Biol Chem **285**(23): 17348-17358.

Mata-Greenwood, E., W. X. Liao, J. Zheng and D. B. Chen (2008). "Differential activation of multiple signalling pathways dictates eNOS upregulation by FGF2 but not VEGF in placental artery endothelial cells." Placenta **29**(8): 708-717.
Matheson, H., J. H W. Veerbeek, D S. Charnock-Jones, G. J. Burton and H. W. Yung (2016). "Morphological and molecular changes in the murine placenta exposed to normobaric hypoxia throughout pregnancy." The JouRNAl of Physiology **594**(5): 1371-1388.
Matlin, A. J., F. Clark and C. W. Smith (2005). "Understanding alteRNAtive splicing: towards a cellular code." Nat Rev Mol Cell Biol **6**(5): 386-398.
Matouskova, M., J. Blazkova, P. Pajer, A. Pavlicek and J. Hejnar (2006). "CpG methylation suppresses transcriptional activity of human syncytin-1 in non-placental tissues." Exp Cell Res **312**(7): 1011-1020.
Matsuura, K., T. Jigami, K. Taniue, Y. Morishita, S. Adachi, T. Senda, A. Nonaka, H. Aburatani, T. Nakamura and T. Akiyama (2011). "Identification of a link between Wnt/beta-catenin signalling and the cell fusion pathway." Nat Commun **2**: 548.
Mayhew, T., D. Charnock Jones, P. Kaufmann, T. Mayhew, D. Charnock Jones and P. Kaufmann (2004). "Aspects of human fetoplacental vasculogenesis and angiogenesis. III. Changes in complicated pregnancies." Placenta **25**: 127-139.
Mayhew, T. M., C. Bowles and F. Yucel (2002). "Hypobaric hypoxia and villous trophoblast: evidence that human pregnancy at high altitude (3600 m) perturbs epithelial turnover and coagulation-fibrinolysis in the intervillous space." Placenta **23**(2-3): 154-162.
Mayhew, T. M., D. S. Charnock-Jones and P. Kaufmann (2004). "Aspects of human fetoplacental vasculogenesis and angiogenesis. III. Changes in complicated pregnancies." Placenta **25**(2-3): 127-139.
Mayhew, T. M., C. Ohadike, P. N. Baker, I. P. Crocker, C. Mitchell and S. S. Ong (2003). "Stereological investigation of placental morphology in pregnancies complicated by pre-eclampsia with and without intrauterine growth restriction." Placenta **24**(2-3): 219-226.
Maynard, S., F. H. Epstein and S. A. Karumanchi (2008). "Preeclampsia and Angiogenic Imbalance." Annual Review of Medicine **59**(1): 61-78.
Maynard, S. E., J.-Y. Min, J. Merchan, K.-H. Lim, J. Li, S. Mondal, T. A. Libermann, J. P. Morgan, F. W. Sellke, I. E. Stillman, F. H. Epstein, V. P. Sukhatme and S. A. Karumanchi (2003). "Excess placental soluble fms-like tyrosine kinase 1 (sFLT-1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia." JouRNAl of Clinical Investigation **111**(5): 649-658.
Maynard, S. E., J.-Y. Min, J. Merchan, K.-H. Lim, J. Li, S. Mondal, T. A. Libermann, J. P. Morgan, F. W. Sellke, I. E. Stillman, F. H. Epstein, V. P. Sukhatme and S. A. Karumanchi (2003). "Excess placental soluble fms-like tyrosine kinase 1 (sFLT-1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia." The JouRNAl of Clinical Investigation **111**(5): 649-658.
Maynard, S. E., S. Venkatesha, R. Thadhani and S. A. Karumanchi (2005). "Soluble Fms-like Tyrosine Kinase 1 and Endothelial Dysfunction in the Pathogenesis of Preeclampsia." Pediatric Research **57**: 1R.
McDonald, L. J. and F. Murad (1996). "Nitric oxide and cyclic GMP signaling." Proc Soc Exp Biol Med **211**(1): 1-6.
McKeeman, G. C., J. E. Ardill, C. M. Caldwell, A. J. Hunter and N. McClure (2004). "Soluble vascular endothelial growth factor receptor-1 (sFLT-1) is increased throughout gestation in patients who have preeclampsia develop." Am J Obstet Gynecol **191**(4): 1240-1246.
McKeown, K. J. and J. R. Challis (2003). "Regulation of 15-hydroxy prostaglandin dehydrogenase by corticotrophin-releasing hormone through a calcium-dependent pathway in human chorion trophoblast cells." J Clin Endocrinol Metab **88**(4): 1737-1741.

McLean, M., A. Bisits, J. Davies, W. Walters, A. Hackshaw, K. De Voss and R. Smith (1999). "Predicting risk of preterm delivery by second-trimester measurement of maternalplasma corticotropin-releasing hormone and α-fetoprotein concentrations." American JouRNAl of Obstetrics & Gynecology **181**(1): 207-215.
McLean, M., A. Bisits, J. Davies, R. Woods, P. Lowry and R. Smith (1995). "A placental clock controlling the length of human pregnancy." Nat Med **1**(5): 460-463.
McLean, M. and R. Smith (1999). "Corticotropin-releasing Hormone in Human Pregnancy and Parturition." Trends Endocrinol Metab **10**(5): 174-178.
McMaster, M. T., Y. Zhou and S. J. Fisher (2004). "Abnormal placentation and the syndrome of preeclampsia." Semin Nephrol **24**(6): 540-547.
Meltzer-Brody, S., A. Stuebe, N. Dole, D. Savitz, D. Rubinow and J. Thorp (2011). "Elevated Corticotropin Releasing Hormone (CRH) during Pregnancy and Risk of Postpartum Depression (PPD)." The JouRNAl of Clinical Endocrinology & Metabolism **96**(1): E40-E47.
Menon, R., C. P. Arora, C. J. Hobel and S. J. Fortunato (2008). "Corticotrophin-releasing hormone in lipopolysaccharide-stimulated term fetal membranes and amniotic fluid from term and preterm birth in African Americans and Caucasians." Reprod Sci **15**(5): 477-483.
Meyer, A. H., C. Ullmer, K. Schmuck, C. Morel, W. Wishart, H. Lübbert and P. Engels (1997). "Localization of the Human CRF2 Receptor to 7p21–p15 by Radiation Hybrid Mapping and FISH Analysis." Genomics **40**(1): 189-190.
Michael, A. E. and A. T. Papageorghiou (2008). "Potential significance of physiological and pharmacological glucocorticoids in early pregnancy." Hum Reprod Update **14**(5): 497-517.
Mighell, A. J., A. F. Markham and P. A. Robinson (1997). "Alu sequences." FEBS Lett **417**(1): 1-5.
Mignot, T. M., B. Paris, B. Carbonne, C. Vauge, F. Ferre and D. Vaiman (2005). "Corticotropin-releasing hormone effects on human pregnant vs. nonpregnant myometrium explants estimated from a mathematical model of uterine contraction." J Appl Physiol (1985) **99**(3): 1157-1163.
Milan, D., J. T. Jeon, C. Looft, V. Amarger, A. Robic, M. Thelander, C. Rogel-Gaillard, S. Paul, N. Iannuccelli, L. Rask, H. Ronne, K. Lundstrom, N. Reinsch, J. Gellin, E. Kalm, P. L. Roy, P. Chardon and L. Andersson (2000). "A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle." Science **288**(5469): 1248-1251.
Million, M., Y. Taché and P. Anton (1999). "Susceptibility of Lewis and Fischer rats to stress-induced worsening of TNB-colitis: protective role of brain CRF." American JouRNAl of Physiology-Gastrointestinal and Liver Physiology **276**(4): G1027-G1036.
Minas, V., U. Jeschke, S. N. Kalantaridou, D. U. Richter, T. Reimer, I. Mylonas, K. Friese and A. Makrigiannakis (2007). "Abortion is associated with increased expression of FasL in decidual leukocytes and apoptosis of extravillous trophoblasts: a role for CRH and urocortin." Molecular Human Reproduction **13**(9): 663-673.
Minas, V., U. Jeschke, S. N. Kalantaridou, D. U. Richter, T. Reimer, I. Mylonas, K. Friese and A. Makrigiannakis (2007). "Abortion is associated with increased expression of FasL in decidual leukocytes and apoptosis of extravillous trophoblasts: a role for CRH and urocortin." Mol Hum Reprod **13**(9): 663-673.
Minet, E., T. Arnould, G. Michel, I. Roland, D. Mottet, M. Raes, J. Remacle and C. Michiels (2000). "ERK activation upon hypoxia: involvement in HIF-1 activation." FEBS Lett **468**(1): 53-58.
Mo, H., D. Ouyang, L. Xu, Q. Gao, X. He, H. Mo, D. Ouyang, L. Xu, Q. Gao and X. He (2013). "Human endogenous retroviral syncytin exerts inhibitory effect on invasive phenotype of B16F10 melanoma cells." Chinese JouRNAl of Cancer Research **25**: 556-564.

Morrish, D. W., D. Bhardwaj, L. K. Dabbagh, H. Marusyk and O. Siy (1987). "Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal human placenta." J Clin Endocrinol Metab **65**(6): 1282-1290.
Mudgett, J. S., J. Ding, L. Guh-Siesel, N. A. Chartrain, L. Yang, S. Gopal and M. M. Shen (2000). "Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis." Proc Natl Acad Sci U S A **97**(19): 10454-10459.
Murakami, M., T. Ichisaka, M. Maeda, N. Oshiro, K. Hara, F. Edenhofer, H. Kiyama, K. Yonezawa and S. Yamanaka (2004). "MTOR Is Essential for Growth and Proliferation in Early Mouse Embryos and Embryonic Stem Cells." Molecular and Cellular Biology **24**(15): 6710-6718.
Muramatsu, Y., K. Fukushima, K. Iino, K. Totsune, K. Takahashi, T. Suzuki, G. Hirasawa, J. Takeyama, M. Ito, M. Nose, A. Tashiro, M. Hongo, Y. Oki, H. Nagura and H. Sasano (2000). "Urocortin and corticotropin-releasing factor receptor expression in the human colonic mucosa." Peptides **21**(12): 1799-1809.
Muramatsu, Y., N. Sugino, T. Suzuki, K. Totsune, K. Takahashi, A. Tashiro, M. Hongo, Y. Oki and H. Sasano (2001). "Urocortin and corticotropin-releasing factor receptor expression in normal cycling human ovaries." J Clin Endocrinol Metab **86**(3): 1362-1369.
Murohara, T., T. Asahara, M. Silver, C. Bauters, H. Masuda, C. Kalka, M. Kearney, D. Chen, J. F. Symes, M. C. Fishman, P. L. Huang and J. M. Isner (1998). "Nitric oxide synthase modulates angiogenesis in response to tissue ischemia." J Clin Invest **101**(11): 2567-2578.
Murohara, T., J. R. Horowitz, M. Silver, Y. Tsurumi, D. Chen, A. Sullivan and J. M. Isner (1998). "Vascular endothelial growth factor/vascular permeability factor enhances vascular permeability via nitric oxide and prostacyclin." Circulation **97**(1): 99-107.
Muttukrishna, S., M. Swer, S. Suri, A. Jamil, J. Calleja-Agius, S. Gangooly, H. Ludlow, D. Jurkovic and E. Jauniaux (2011). "Soluble FLT-1 and PLGF: New Markers of Early Pregnancy Loss?" PLoS ONE **6**(3): e18041.
Nagamatsu, T., T. Fujii, M. Kusumi, L. Zou, T. Yamashita, Y. Osuga, M. Momoeda, S. Kozuma and Y. Taketani (2004). "Cytotrophoblasts up-regulate soluble fms-like tyrosine kinase-1 expression under reduced oxygen: an implication for the placental vascular development and the pathophysiology of preeclampsia." Endocrinology **145**(11): 4838-4845.
Nagashima, K., H. Yagi, H. Yunoki, T. Noji and T. Kuroume (1987). "Cord blood levels of corticotropin-releasing factor." Biol Neonate **51**(1): 1-4.
Nelson, D. M., R. D. Johnson, S. D. Smith, E. Y. Anteby and Y. Sadovsky (1999). "Hypoxia limits differentiation and up-regulates expression and activity of prostaglandin H synthase 2 in cultured trophoblast from term human placenta." American JouRNAl of Obstetrics and Gynecology **180**(4): 896-902.
Nemoto, T., A. Mano-Otagiri and T. Shibasaki (2005). "Urocortin 2 induces tyrosine hydroxylase phosphorylation in PC12 cells." Biochem Biophys Res Commun **330**(3): 821-831.
Ng, E. K., T. N. Leung, N. B. Tsui, T. K. Lau, N. S. Panesar, R. W. Chiu and Y. M. Lo (2003). "The concentration of circulating corticotropin-releasing hormone mRNA in maternalplasma is increased in preeclampsia." Clin Chem **49**(5): 727-731.
Ni, X., Y. Hou, B. R. King, X. Tang, M. A. Read, R. Smith and R. C. Nicholson (2004). "Estrogen receptor-mediated down-regulation of corticotropin-releasing hormone gene expression is dependent on a cyclic adenosine 3',5'-monophosphate regulatory element in human placental syncytiotrophoblast cells." J Clin Endocrinol Metab **89**(5): 2312-2318.
Ni, X., Y. Hou, R. Yang, X. Tang, R. Smith and R. C. Nicholson (2004). "Progesterone receptors A and B differentially modulate corticotropin-releasing hormone gene expression through a cAMP regulatory element." Cell Mol Life Sci **61**(9): 1114-1122.

Nodwell, A., L. Carmichael, M. Fraser, J. Challis and B. Richardson (1999). "Placental release of corticotrophin-releasing hormone across the umbilical circulation of the human newborn." Placenta **20**(2-3): 197-202.
Nohara, A., M. Ohmichi, K. Koike, N. Masumoto, M. Kobayashi, M. Akahane, H. Ikegami, K. Hirota, A. Miyake and Y. Murata (1996). "The role of mitogen-activated protein kinase in oxytocin-induced contraction of uterine smooth muscle in pregnant rat." Biochem Biophys Res Commun **229**(3): 938-944.
Nolten, W. E., M. D. Lindheimer, P. A. Rueckert, S. Oparil and E. N. Ehrlich (1980). "DiuRNAl Patterns and Regulation of Cortisol Secretion in Pregnancy*." The JouRNAl of Clinical Endocrinology & Metabolism **51**(3): 466-472.
Noorali, S., I. C. Rotar, C. Lewis, J. P. Pestaner, D. G. Pace, A. Sison and O. Bagasra (2009). "Role of HERV-W syncytin-1 in placentation and maintenance of human pregnancy." Appl Immunohistochem Mol Morphol **17**(4): 319-328.
Nulsen, J. C., S. L. Silavin, L. C. Kao, G. E. Ringler, H. J. Kliman and J. F. Strauss, 3rd (1989). "Control of the steroidogenic machinery of the human trophoblast by cyclic AMP." J Reprod Fertil Suppl **37**: 147-153.
Nyfeler, B., V. Reiterer, M. W. Wendeler, E. Stefan, B. Zhang, S. W. Michnick and H.-P. Hauri (2008). "Identification of ERGIC-53 as an intracellular transport receptor of α₁-antitrypsin." The JouRNAl of Cell Biology **180**(4): 705-712.
Ohmichi, M., K. Koike, A. Kimura, K. Masuhara, H. Ikegami, Y. Ikebuchi, T. Kanzaki, K. Touhara, M. Sakaue, Y. Kobayashi, M. Akabane, A. Miyake and Y. Murata (1997). "Role of Mitogen-Activated Protein Kinase Pathway in Prostaglandin F2α-Induced Rat Puerperal Uterine Contraction." Endocrinology **138**(8): 3103-3111.
Okada, Y., Y. Ueshin, A. Isotani, T. Saito-Fujita, H. Nakashima, K. Kimura, A. Mizoguchi, M. Oh-Hora, Y. Mori, M. Ogata, R. G. Oshima, M. Okabe and M. Ikawa (2007). "Complementation of placental defects and embryonic lethality by trophoblast-specific lentiviral gene transfer." Nat Biotechnol **25**(2): 233-237.
Orendi, K., M. Gauster, G. Moser, H. Meiri and B. Huppertz (2010). "The choriocarcinoma cell line BEWO: syncytial fusion and expression of syncytium-specific proteins." Reproduction **140**(5): 759-766.
Orendi, K., V. Kivity, M. Sammar, Y. Grimpel, R. Gonen, H. Meiri, E. Lubzens and B. Huppertz (2011). "Placental and trophoblastic in vitro models to study preventive and therapeutic agents for preeclampsia." Placenta **32 Suppl**: S49-54.
Palmer, K. R., S. Tong and T. J. Kaitu'u-Lino (2017). "Placental-specific sFLT-1: role in pre-eclamptic pathophysiology and its translational possibilities for clinical prediction and diagnosis." MHR: Basic science of reproductive medicine **23**(2): 69-78.
Palmer, S. K., L. G. Moore, D. Young, B. Cregger, J. C. Berman and S. Zamudio (1999). "Altered blood pressure course during normal pregnancy and increased preeclampsia at high altitude (3100 meters) in Colorado." Am J Obstet Gynecol **180**(5): 1161-1168.
Papadopoulou, N., J. Chen, H. S. Randeva, M. A. Levine, E. W. Hillhouse and D. K. Grammatopoulos (2004). "Protein kinase A-induced negative regulation of the corticotropin-releasing hormone R1alpha receptor-extracellularly regulated kinase signal transduction pathway: the critical role of Ser301 for signaling switch and selectivity." Mol Endocrinol **18**(3): 624-639.
Park, J. E., H. H. Chen, J. Winer, K. A. Houck and N. Ferrara (1994). "Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to FLT-1 but not to Flk-1/KDR." JouRNAl of Biological Chemistry **269**(41): 25646-25654.

Pasquarette, M. M., P. M. Stewart, M. L. Ricketts, K. Imaishi and J. I. Mason (1996). "Regulation of 11β-hydroxysteroid dehydrogenase type 2 activity and mRNA in human choriocarcinoma cells." JouRNAl of Molecular Endocrinology **16**(3): 269-275.
Patel, J., K. Landers, R. H. Mortimer and K. Richard (2010). "Regulation of hypoxia inducible factors (HIF) in hypoxia and normoxia during placental development." Placenta **31**(11): 951-957.
Payne, S. G., S. C. Smith, S. T. Davidge, P. N. Baker and L. J. Guilbert (1999). "Death receptor Fas/Apo-1/CD95 expressed by human placental cytotrophoblasts does not mediate apoptosis." Biol Reprod **60**(5): 1144-1150.
Pelham, H. R. B. and J. E. Rothman (2000). "The Debate about Transport in the Golgi—Two Sides of the Same Coin?" Cell **102**(6): 713-719.
PERKins, A. V., F. Eben, C. D. Wolfe, H. M. Schulte and E. A. Linton (1993). "Plasma measurements of corticotrophin-releasing hormone-binding protein in normal and abnormal human pregnancy." J Endocrinol **138**(1): 149-157.
PERKins, A. V., E. A. Linton, F. Eben, J. Simpson, C. D. Wolfe and C. W. Redman (1995). "Corticotrophin-releasing hormone and corticotrophin-releasing hormone binding protein in normal and pre-eclamptic human pregnancies." Br J Obstet Gynaecol **102**(2): 118-122.
PERKins, A. V., E. A. Linton, F. Eben, J. Simpson, C. D. A. Wolfe and C. W. G. Redman (1995). "Corticotrophin-releasing hormone and corticotrophin- releasing hormone binding protein in normal and pre-eclamptic human pregnancies." BJOG: An InteRNAtional JouRNAl of Obstetrics & Gynaecology **102**(2): 118-122.
PERKins, A. V., C. D. Wolfe, F. Eben, P. Soothill and E. A. Linton (1995). "Corticotrophin-releasing hormone-binding protein in human fetal plasma." J Endocrinol **146**(3): 395-401.
Perrin, M. H., C. J. Donaldson, R. Chen, K. A. Lewis and W. W. Vale (1993). "Cloning and functional expression of a rat brain corticotropin releasing factor (CRF) receptor." Endocrinology **133**(6): 3058-3061.
Perrin, M. H., S. Sutton, D. L. Bain, W. T. Berggren and W. W. Vale (1998). "The first extracellular domain of corticotropin releasing factor-R1 contains major binding determinants for urocortin and astressin." Endocrinology **139**(2): 566-570.
Petraglia, F., L. Aguzzoli, P. Florio, P. Baumann, A. D. Genazzani, C. Di Carlo and R. Romero (1995). "Maternalplasma and placental immunoreactive corticotrophin-releasing factor concentrations in infection-associated term and pre-term delivery." Placenta **16**(2): 157-164.
Petraglia, F., P. Florio, C. Benedetto, L. Marozio, A. M. Di Blasio, C. Ticconi, E. Piccione, S. Luisi, A. R. Genazzani and W. Vale (1999). "Urocortin stimulates placental adrenocorticotropin and prostaglandin release and myometrial contractility in vitro." J Clin Endocrinol Metab **84**(4): 1420-1423.
Petraglia, F., P. Florio, R. Gallo, C. Salvestroni, M. Lombardo, A. D. Genazzani, C. Di Carlo, M. Stomati, G. D'Ambrogio and P. G. Artini (1996). "Corticotropin-releasing factor-binding protein: origins and possible functions." Horm Res **45**(3-5): 187-191.
Petraglia, F., P. Florio, R. Gallo, T. Simoncini, M. Saviozzi, A. M. Di Blasio, J. Vaughan and W. Vale (1996). "Human placenta and fetal membranes express human urocortin mRNA and peptide." J Clin Endocrinol Metab **81**(10): 3807-3810.
Petraglia, F., P. Florio, T. Simoncini, R. J. Woods, A. Giuntini, R. Gremigni, G. B. Serra, A. R. Genazzani and P. J. Lowry (1997). "Cord plasma corticotropin-releasing factor-binding protein (CRF-BP) in term and preterm labour." Placenta **18**(2-3): 115-119.
Petraglia, F., G. C. Garuti, B. De Ramundo, S. Angioni, A. R. Genazzani and L. M. Bilezikjian (1990). "Mechanism of action of interleukin-1 beta in increasing corticotropin-releasing factor and adrenocorticotropin hormone release from cultured human placental cells." Am J Obstet Gynecol **163**(4 Pt 1): 1307-1312.

Petraglia, F., A. Imperatore and J. R. Challis (2010). "Neuroendocrine mechanisms in pregnancy and parturition." Endocr Rev **31**(6): 783-816.
Petraglia, F., E. Potter, V. A. Cameron, S. Sutton, D. P. Behan, R. J. Woods, P. E. Sawchenko, P. J. Lowry and W. Vale (1993). "Corticotropin-releasing factor-binding protein is produced by human placenta and intrauterine tissues." J Clin Endocrinol Metab **77**(4): 919-924.
Petraglia, F., P. E. Sawchenko, J. Rivier and W. Vale (1987). "Evidence for local stimulation of ACTH secretion by corticotropin-releasing factor in human placenta." Nature **328**(6132): 717-719.
Petraglia, F., S. Sutton and W. Vale (1989). "Neurotransmitters and peptides modulate the release of immunoreactive corticotropin-releasing factor from cultured human placental cells." Am J Obstet Gynecol **160**(1): 247-251.
Petraglia, F., S. Tabanelli, M. C. Galassi, G. C. Garuti, A. C. Mancini, A. R. Genazzani and E. Gurpide (1992). "Human decidua and in vitro decidualized endometrial stromal cells at term contain immunoreactive corticotropin-releasing factor (CRF) and CRF messenger ribonucleic acid." J Clin Endocrinol Metab **74**(6): 1427-1431.
Petsas, G., U. Jeschke, D. U. Richter, V. Minas, A. Hammer, S. Kalantaridou, B. Toth, C. Tsatsanis, K. Friese and A. Makrigiannakis (2012). "Aberrant expression of corticotropin-releasing hormone in pre-eclampsia induces expression of FasL in maternalmacrophages and extravillous trophoblast apoptosis." Mol Hum Reprod **18**(11): 535-545.
Phillips, T. A., J. Ni, G. Pan, S. M. Ruben, Y. F. Wei, J. L. Pace and J. S. Hunt (1999). "TRAIL (Apo-2L) and TRAIL receptors in human placentas: implications for immune privilege." J Immunol **162**(10): 6053-6059.
Pijnenborg, R., E. Ball, J. N. Bulmer, M. Hanssens, S. C. Robson and L. Vercruysse (2006). "In vivo analysis of trophoblast cell invasion in the human." Methods Mol Med **122**: 11-44.
Pijnenborg, R., J. M. Bland, W. B. Robertson, G. Dixon and I. Brosens (1981). "The pattern of interstitial trophoblastic invasion of the myometrium in early human pregnancy." Placenta **2**(4): 303-316.
Pisarchik, A. and A. T. Slominski (2001). "AlteRNAtive splicing of CRH-R1 receptors in human and mouse skin: identification of new variants and their differential expression." Faseb j **15**(14): 2754-2756.
Plate, K. H., G. Breier, H. A. Weich and W. Risau (1992). "Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo." Nature **359**: 845.
Pollheimer, J. and M. Knofler (2005). "Signalling pathways regulating the invasive differentiation of human trophoblasts: a review." Placenta **26 Suppl A**: S21-30.
Poon, L. C., E. Zaragoza, R. Akolekar, E. Anagnostopoulos and K. H. Nicolaides (2008). "Maternalserum placental growth factor (PLGF) in small for gestational age pregnancy at 11(+0) to 13(+6) weeks of gestation." Prenat Diagn **28**(12): 1110-1115.
Pötgens, A. J. G., S. Drewlo, M. Kokozidou and P. Kaufmann (2004). "Syncytin: the major regulator of trophoblast fusion? Recent developments and hypotheses on its action." Human Reproduction Update **10**(6): 487-496.
Pringle, K. G., K. L. Kind, A. N. Sferruzzi-Perri, J. G. Thompson and C. T. Roberts (2010). "Beyond oxygen: complex regulation and activity of hypoxia inducible factors in pregnancy." Human Reproduction Update **16**(4): 415-431.
Prinster, S. C., C. Hague and R. A. Hall (2005). "Heterodimerization of g protein-coupled receptors: specificity and functional significance." Pharmacol Rev **57**(3): 289-298.
Pugazhenthi, S., A. Nesterova, C. Sable, K. A. Heidenreich, L. M. Boxer, L. E. Heasley and J. E.-B. Reusch (2000). "AKT/Protein Kinase B Up-regulates Bcl-2 Expression through cAMP-response Element-binding Protein." JouRNAl of Biological Chemistry **275**(15): 10761-10766.

Qiu, Y., C. Hoareau-Aveilla, S. Oltean, S. J. Harper and D. O. Bates (2009). "The anti-angiogenic isoforms of VEGF in health and disease." Biochemical Society transactions **37**(Pt 6): 1207-1213.
Rademaker, M. T., C. J. Charles, E. A. Espiner, S. Fisher, C. M. Frampton, C. M. Kirkpatrick, J. G. Lainchbury, M. G. Nicholls, A. M. Richards and W. W. Vale (2002). "Beneficial hemodynamic, endocrine, and renal effects of urocortin in experimental heart failure: comparison with normal sheep." J Am Coll Cardiol **40**(8): 1495-1505.
Rahimi, N., V. Dayanir and K. Lashkari (2000). "Receptor chimeras indicate that the vascular endothelial growth factor receptor-1 (VEGFR-1) modulates mitogenic activity of VEGFR-2 in endothelial cells." J Biol Chem **275**(22): 16986-16992.
Ratsep, M. T., P. Carmeliet, M. A. Adams and B. A. Croy (2014). "Impact of placental growth factor deficiency on early mouse implant site angiogenesis." Placenta **35**(9): 772-775.
Ravindranath, N., L. Little-Ihrig, H. S. Phillips, N. Ferrara and A. J. Zeleznik (1992). "Vascular endothelial growth factor messenger ribonucleic acid expression in the primate ovary." Endocrinology **131**(1): 254-260.
Red-Horse, K., Y. Zhou, O. Genbacev, A. Prakobphol, R. Foulk, M. McMaster and S. J. Fisher (2004). "Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface." J Clin Invest **114**(6): 744-754.
Redman, C. W. and I. L. Sargent (2005). "Latest advances in understanding preeclampsia." Science **308**(5728): 1592-1594.
Rehman, G., A. Shehzad, A. L. Khan and M. Hamayun (2014). "Role of AMP-activated protein kinase in cancer therapy." Arch Pharm (Weinheim) **347**(7): 457-468.
Rehman, K. S., R. Sirianni, C. R. Parker, Jr., W. E. Rainey and B. R. Carr (2007). "The regulation of adrenocorticotrophic hormone receptor by corticotropin-releasing hormone in human fetal adrenal definitive/transitional zone cells." Reprod Sci **14**(6): 578-587.
Reshef, E., Z. M. Lei, C. V. Rao, D. D. Pridham, N. Chegini and J. L. Luborsky (1990). "The Presence of Gonadotropin Receptors in Nonpregnant Human Uterus, Human Placenta, Fetal Membranes, and Decidua*." The JouRNAl of Clinical Endocrinology & Metabolism **70**(2): 421-430.
Reshetnikova, O. S., G. J. Burton, A. P. Milovanov and E. I. Fokin (1996). "Increased incidence of placental chorioangioma in high-altitude pregnancies: Hypobaric hypoxia as a possible etiologic factor." American JouRNAl of Obstetrics & Gynecology **174**(2): 557-561.
Reyes, T. M., K. Lewis, M. H. Perrin, K. S. Kunitake, J. Vaughan, C. A. Arias, J. B. Hogenesch, J. Gulyas, J. Rivier, W. W. Vale and P. E. Sawchenko (2001). "Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors." Proc Natl Acad Sci U S A **98**(5): 2843-2848.
Reynolds, L. P., J. S. Caton, D. A. Redmer, A. T. Grazul-Bilska, K. A. Vonnahme, P. P. Borowicz, J. S. Luther, J. M. Wallace, G. Wu and T. E. Spencer (2006). "Evidence for altered placental blood flow and vascularity in compromised pregnancies." J Physiol **572**(Pt 1): 51-58.
Reynolds, L. P. and D. A. Redmer (2001). "Angiogenesis in the placenta." Biol Reprod **64**(4): 1033-1040.
Rhee, S. H., E. L. Ma, Y. Lee, Y. Tache, C. Pothoulakis and E. Im (2015). "Corticotropin Releasing Hormone and Urocortin 3 Stimulate Vascular Endothelial Growth Factor Expression through the cAMP/CREB Pathway." J Biol Chem **290**(43): 26194-26203.
Rhee, S. H., E. L. Ma, Y. Lee, Y. Taché, C. Pothoulakis and E. Im (2015). "Corticotropin releasing hormone and urocortin 3 stimulate vascular endothelial growth factor expression through the cAMP/CREB pathway." JouRNAl of Biological Chemistry.
Rhee, S. H., E. L. Ma, Y. Lee, Y. Taché, C. Pothoulakis and E. Im (2015). "Corticotropin Releasing Hormone and Urocortin 3 Stimulate Vascular Endothelial Growth Factor

Expression through the cAMP/CREB Pathway." JouRNAl of Biological Chemistry **290**(43): 26194-26203.
Richards, J. S. (2001). "New Signaling Pathways for Hormones and Cyclic Adenosine 3′,5′-Monophosphate Action in Endocrine Cells." Molecular Endocrinology **15**(2): 209-218.
Riley, S. C., J. C. Walton, J. M. Herlick and J. R. Challis (1991). "The localization and distribution of corticotropin-releasing hormone in the human placenta and fetal membranes throughout gestation." J Clin Endocrinol Metab **72**(5): 1001-1007.
Ringler, G. E., K. Lee-Chuan, W. L. Miller and J. F. Strauss (1989). "Effects of 8-bromo-cAMP on expression of endocrine functions by cultured human trophoblast cells. Regulation of specific mRNAs." Molecular and Cellular Endocrinology **61**(1): 13-21.
Ringler, G. E. and J. F. Strauss, 3rd (1990). "In vitro systems for the study of human placental endocrine function." Endocr Rev **11**(1): 105-123.
Robinson, B. G., R. L. Emanuel, D. M. Frim and J. A. Majzoub (1988). "Glucocorticoid stimulates expression of corticotropin-releasing hormone gene in human placenta." Proc Natl Acad Sci U S A **85**(14): 5244-5248.
Roche, P. J., R. J. Crawford, R. T. Fernley, G. W. Tregear and J. P. Coghlan (1988). "Nucleotide sequence of the gene coding for ovine corticotropin-releasing factor and regulation of its mRNA levels by glucocorticoids." Gene **71**(2): 421-431.
Rodesch, F., P. Simon, C. Donner and E. Jauniaux (1992). "Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy." Obstet Gynecol **80**(2): 283-285.
Rodway, M., F. Z. Zhou, J. Benoit, B. H. Yuen and P. C. Leung (1988). "Differential effects of 8-bromo-cyclic AMP on human chorionic gonadotropin (hCG), progesterone and estrogen production by term placental cells." Life Sci **43**(18): 1451-1458.
Rohde, E., J. Furkert, K. Fechner, M. Beyermann, M. J. Mulvany, R. M. Richter, C. Denef, M. Bienert and H. Berger (1996). "Corticotropin-releasing hormone (CRH) receptors in the mesenteric small arteries of rats resemble the (2)-subtype." Biochem Pharmacol **52**(6): 829-833.
Roos, S., N. Jansson, I. Palmberg, K. Säljö, T. L. Powell and T. Jansson (2007). "Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth." The JouRNAl of Physiology **582**(Pt 1): 449-459.
Ross, P. C., C. M. Kostas and T. V. Ramabhadran (1994). "A variant of the human corticotropin-releasing factor (CRF) receptor: cloning, expression and pharmacology." Biochem Biophys Res Commun **205**(3): 1836-1842.
Runic, R., C. J. Lockwood, L. LaChapelle, B. Dipasquale, R. I. Demopoulos, A. Kumar and S. Guller (1998). "Apoptosis and Fas expression in human fetal membranes." J Clin Endocrinol Metab **83**(2): 660-666.
Runic, R., C. J. Lockwood, Y. Ma, B. Dipasquale and S. Guller (1996). "Expression of Fas ligand by human cytotrophoblasts: implications in placentation and fetal survival." J Clin Endocrinol Metab **81**(8): 3119-3122.
Saeed, B., M. Fawcett and C. Self (2001). "Corticotropin-releasing hormone binding to the syncytiotrophoblast membranes." Eur J Clin Invest **31**(2): 125-130.
Saffer, C., G. Olson, K. A. Boggess, R. Beyerlein, C. Eubank and B. M. Sibai (2013). "Determination of placental growth factor (PLGF) levels in healthy pregnant women without signs or symptoms of preeclampsia." Pregnancy Hypertens **3**(2): 124-132.
Saijonmaa, O., T. Laatikainen and T. Wahlstrom (1988). "Corticotrophin-releasing factor in human placenta: localization, concentration and release in vitro." Placenta **9**(4): 373-385.
Sandman, C. A., L. Glynn, C. D. Schetter, P. Wadhwa, T. Garite, A. Chicz-DeMet and C. Hobel (2006). "Elevated maternalcortisol early in pregnancy predicts third trimester levels of

placental corticotropin releasing hormone (CRH): priming the placental clock." Peptides **27**(6): 1457-1463.
Sasaki, A., O. Shinkawa and K. Yoshinaga (1989). "Placental corticotropin-releasing hormone may be a stimulator of maternalpituitary adrenocorticotropic hormone secretion in humans." J Clin Invest **84**(6): 1997-2001.
Sasaki, A., P. Tempst, A. S. Liotta, A. N. Margioris, L. E. Hood, S. B. Kent, S. Sato, O. Shinkawa, K. Yoshinaga and D. T. Krieger (1988). "Isolation and characterization of a corticotropin-releasing hormone-like peptide from human placenta." J Clin Endocrinol Metab **67**(4): 768-773.
Sato, T. N., Y. Tozawa, U. Deutsch, K. Wolburg-Buchholz, Y. Fujiwara, M. Gendron-Maguire, T. Gridley, H. Wolburg, W. Risau and Y. Qin (1995). "Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation." Nature **376**(6535): 70-74.
Scatena, C. D. and S. Adler (1998). "Characterization of a human-specific regulator of placental corticotropin-releasing hormone." Mol Endocrinol **12**(8): 1228-1240.
Schekman, R. and L. Orci (1996). "Coat Proteins and Vesicle Budding." Science **271**(5255): 1526-1533.
Schoof, E., M. Girstl, W. Frobenius, M. Kirschbaum, H. G. Dorr, W. Rascher and J. Dotsch (2001). "Decreased gene expression of 11beta-hydroxysteroid dehydrogenase type 2 and 15-hydroxyprostaglandin dehydrogenase in human placenta of patients with preeclampsia." J Clin Endocrinol Metab **86**(3): 1313-1317.
Seckl, J. R. (2004). "11beta-hydroxysteroid dehydrogenases: changing glucocorticoid action." Curr Opin Pharmacol **4**(6): 597-602.
Seckl, J. R., M. Cleasby and M. J. Nyirenda (2000). "Glucocorticoids, 11beta-hydroxysteroid dehydrogenase, and fetal programming." Kidney Int **57**(4): 1412-1417.
Segre, G. V. and S. R. Goldring (1993). "Receptors for secretin, calcitonin, parathyroid hormone (PTH)/PTH-related peptide, vasoactive intestinal peptide, glucagonlike peptide 1, growth hormone-releasing hormone, and glucagon belong to a newly discovered G-protein-linked receptor family." Trends Endocrinol Metab **4**(10): 309-314.
Sela, S., A. Itin, S. Natanson-Yaron, C. Greenfield, D. Goldman-Wohl, S. Yagel and E. Keshet (2008). "A novel human-specific soluble vascular endothelial growth factor receptor 1: cell-type-specific splicing and implications to vascular endothelial growth factor homeostasis and preeclampsia." Circ Res **102**(12): 1566-1574.
Semenza, G. L. (2010). "HIF-1: upstream and downstream of cancer metabolism." Curr Opin Genet Dev **20**(1): 51-56.
Senger, D., S. Galli, A. Dvorak, C. Perruzzi, V. Harvey and H. Dvorak (1983). "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid." Science **219**(4587): 983-985.
Senger, D. R., S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey and H. F. Dvorak (1983). "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid." Science **219**(4587): 983-985.
Sengupta, S., T. R. Peterson and D. M. Sabatini (2010). "Regulation of the MTOR complex 1 pathway by nutrients, growth factors, and stress." Mol Cell **40**(2): 310-322.
Shaywitz, A. J. and M. E. Greenberg (1999). "CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals." Annu Rev Biochem **68**: 821-861.
Shen, H., M. Clauss, J. Ryan, A. M. Schmidt, P. Tijburg, L. Borden, D. Connolly, D. Stern and J. Kao (1993). "Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes." Blood **81**(10): 2767-2773.
Sherer, D. M. (1998). "First trimester ultrasonography of multiple gestations: a review." Obstet Gynecol Surv **53**(11): 715-726.

Shi, Q. J., Z. M. Lei, C. V. Rao and J. Lin (1993). "Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts." Endocrinology **132**(3): 1387-1395.
Shi, Q. J., Z. M. Lei, C. V. Rao and J. Lin (1993). "Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts." Endocrinology **132**(3): 1387-1395.
Shibahara, S., Y. Morimoto, Y. Furutani, M. Notake, H. Takahashi, S. Shimizu, S. Horikawa and S. Numa (1983). "Isolation and sequence analysis of the human corticotropin-releasing factor precursor gene." Embo j **2**(5): 775-779.
Shibasaki, T., E. Odagiri, K. Shizume and N. Ling (1982). "Corticotropin-releasing factor-like activity in human placental extracts." J Clin Endocrinol Metab **55**(2): 384-386.
Shibata, E., A. Rajakumar, R. W. Powers, R. W. Larkin, C. Gilmour, L. M. Bodnar, W. R. Crombleholme, R. B. Ness, J. M. Roberts and C. A. Hubel (2005). "Soluble fms-like tyrosine kinase 1 is increased in preeclampsia but not in normotensive pregnancies with small-for-gestational-age neonates: relationship to circulating placental growth factor." J Clin Endocrinol Metab **90**(8): 4895-4903.
Shibuya, M., S. Yamaguchi, A. Yamane, T. Ikeda, A. Tojo, H. Matsushime and M. Sato (1990). "Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (FLT) closely related to the fms family." Oncogene **5**(4): 519-524.
Shiu, Y., J. Weiss, J. Hoying, M. Iwamoto, I. Joung, C. Quam, Y. Shiu, J. Weiss, J. Hoying, M. Iwamoto, I. Joung and C. Quam (2005). "The role of mechanical stresses in angiogenesis." Critical Reviews in Biomedical Engineering **33**: 431-510.
Shu, C., Z. Liu, L. Cui, C. Wei, S. Wang, J. J. Tang, M. Cui, G. Lian, W. Li, X. Liu, H. Xu, J. Jiang, P. Lee, D. Y. Zhang, J. He and F. Ye (2014). "Protein profiling of preeclampsia placental tissues." PLoS One **9**(11): e112890.
Shu, Q., W. Li, J. Li, W. Wang, C. Liu and K. Sun (2014). "Cross-talk between cAMP and MAPK pathways in HSD11B2 induction by hCG in placental trophoblasts." PLoS One **9**(9): e107938.
Simoncini, T., R. Apa, F. M. Reis, F. Miceli, M. Stomati, L. Driul, A. Lanzone, A. R. Genazzani and F. Petraglia (1999). "Human umbilical vein endothelial cells: a new source and potential target for corticotropin-releasing factor." J Clin Endocrinol Metab **84**(8): 2802-2806.
Sirianni, R., B. A. Mayhew, B. R. Carr, C. R. Parker, Jr. and W. E. Rainey (2005). "Corticotropin-releasing hormone (CRH) and urocortin act through type 1 CRH receptors to stimulate dehydroepiandrosterone sulfate production in human fetal adrenal cells." J Clin Endocrinol Metab **90**(9): 5393-5400.
Skelton, K. H., M. J. Owens and C. B. Nemeroff (2000). "The neurobiology of urocortin." Regul Pept **93**(1-3): 85-92.
Slominski, A. and J. Wortsman (2000). "Neuroendocrinology of the skin." Endocr Rev **21**(5): 457-487.
Smith, G. W., J. M. Aubry, F. Dellu, A. Contarino, L. M. Bilezikjian, L. H. Gold, R. Chen, Y. Marchuk, C. Hauser, C. A. Bentley, P. E. Sawchenko, G. F. Koob, W. Vale and K. F. Lee (1998). "Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development." Neuron **20**(6): 1093-1102.
Smith, R. (2007). "Parturition." N Engl J Med **356**(3): 271-283.
Smith, R., J. I. Smith, X. Shen, P. J. Engel, M. E. Bowman, S. A. McGrath, A. M. Bisits, P. McElduff, W. B. Giles and D. W. Smith (2009). "Patterns of plasma corticotropin-releasing hormone, progesterone, estradiol, and estriol change and the onset of human labor." J Clin Endocrinol Metab **94**(6): 2066-2074.

Soothill, P. W., K. H. Nicolaides, C. H. Rodeck and S. CAMPbell (1986). "Effect of gestational age on fetal and intervillous blood gas and acid-base values in human pregnancy." Fetal Ther **1**(4): 168-175.
Soygur, B., L. Sati, R. Demir, B. Soygur, L. Sati and R. Demir (2016). "Altered expression of human endogenous retroviruses syncytin-1, syncytin-2 and their receptors in human normal and gestational diabetic placenta." Histol Histopathol **31**: 1037-1047.
Stengel, A. and Y. Taché (2010). "Corticotropin-releasing factor signaling and visceral response to stress." Experimental Biology and Medicine **235**(10): 1168-1178.
Stepan, H., I. Herraiz, D. Schlembach, S. Verlohren, S. Brennecke, F. Chantraine, E. Klein, O. Lapaire, E. Llurba, A. Ramoni, M. Vatish, D. Wertaschnigg and A. Galindo (2015). "Implementation of the sFLT-1/PLGF ratio for prediction and diagnosis of pre-eclampsia in singleton pregnancy: implications for clinical practice." Ultrasound in Obstetrics & Gynecology **45**(3): 241-246.
Straszewski-Chavez, S. L., V. M. Abrahams and G. Mor (2005). "The role of apoptosis in the regulation of trophoblast survival and differentiation during pregnancy." Endocr Rev **26**(7): 877-897.
Stuehr, D. J. and M. A. Marletta (1985). "Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide." Proc Natl Acad Sci U S A **82**(22): 7738-7742.
Suda, T., N. Tomori, F. Tozawa, H. Demura, K. Shizume, T. Mouri, Y. Miura and N. Sasano (1984). "Immunoreactive corticotropin and corticotropin-releasing factor in human hypothalamus, adrenal, lung cancer, and pheochromocytoma." J Clin Endocrinol Metab **58**(5): 919-924.
Sun, K., K. Yang and J. R. Challis (1998). "Regulation of 11beta-hydroxysteroid dehydrogenase type 2 by progesterone, estrogen, and the cyclic adenosine 5'-monophosphate pathway in cultured human placental and chorionic trophoblasts." Biol Reprod **58**(6): 1379-1384.
Suri, C., J. McClain, G. Thurston, D. M. McDonald, H. Zhou, E. H. Oldmixon, T. N. Sato and G. D. Yancopoulos (1998). "Increased Vascularization in Mice Overexpressing Angiopoietin-1." Science **282**(5388): 468-471.
Suzuki, H., M. Kawasaki, H. Ohnishi, T. Nakamura and Y. Ueta (2009). "Regulatory mechanism of the arginine vasopressin-enhanced green fluorescent protein fusion gene expression in acute and chronic stress." Peptides **30**(9): 1763-1770.
Swanson, L. W., P. E. Sawchenko, R. W. Lind and J. H. Rho (1987). "The CRH motoneuron: differential peptide regulation in neurons with possible synaptic, paracrine, and endocrine outputs." Ann N Y Acad Sci **512**: 12-23.
Taché, Y. and B. Bonaz (2007). "Corticotropin-releasing factor receptors and stress-related alterations of gut motor function." The JouRNAl of Clinical Investigation **117**(1): 33-40.
Takahashi, A., Y. Kureishi, J. Yang, Z. Luo, K. Guo, D. Mukhopadhyay, Y. Ivashchenko, D. Branellec and K. Walsh (2002). "Myogenic AKT Signaling Regulates Blood Vessel Recruitment during Myofiber Growth." Molecular and Cellular Biology **22**(13): 4803-4814.
Takahashi, S. (2011). "Vascular Endothelial Growth Factor (VEGF), VEGF Receptors and Their Inhibitors for Antiangiogenic Tumor Therapy." Biological and Pharmaceutical Bulletin **34**(12): 1785-1788.
Tal, R. (2012). "The role of hypoxia and hypoxia-inducible factor-1alpha in preeclampsia pathogenesis." Biol Reprod **87**(6): 134.
Tanaka, Y., S. Makino, T. Noguchi, K. Tamura, T. Kaneda and K. Hashimoto (2003). "Effect of stress and adrenalectomy on urocortin II mRNA expression in the hypothalamic paraventricular nucleus of the rat." Neuroendocrinology **78**(1): 1-11.

Tao, J. and S. Li (2005). "Effects of urocortin via ion mechanisms or CRF receptors?" Biochem Biophys Res Commun **336**(3): 731-736.
Tayade, C., D. Hilchie, H. He, Y. Fang, L. Moons, P. Carmeliet, R. A. Foster and B. A. Croy (2007). "Genetic deletion of placenta growth factor in mice alters uterine NK cells." J Immunol **178**(7): 4267-4275.
Teli, T., D. Markovic, M. E. Hewitt, M. A. Levine, E. W. Hillhouse and D. K. Grammatopoulos (2008). "Structural domains determining signalling characteristics of the CRH-receptor type 1 variant R1beta and response to PKC phosphorylation." Cell Signal **20**(1): 40-49.
Tertemiz, F., U. Kayisli, A. Arici, R. Demir, F. Tertemiz, U. Kayisli, A. Arici and R. Demir (2005). "Apoptosis contributes to vascular lumen formation and vascular branching in human placental vasculogenesis." Biology of Reproduction **72**: 727-735.
Thadhani, R., T. Kisner, H. Hagmann, V. Bossung, S. Noack, W. Schaarschmidt, A. Jank, A. Kribs, O. A. Cornely, C. Kreyssig, L. Hemphill, A. C. Rigby, S. Khedkar, T. H. Lindner, P. Mallmann, H. Stepan, S. A. Karumanchi and T. Benzing (2011). "Pilot study of extracorporeal removal of soluble fms-like tyrosine kinase 1 in preeclampsia." Circulation **124**(8): 940-950.
Thomas, C. P., J. I. Andrews and K. Z. Liu (2007). "Intronic polyadenylation signal sequences and alteRNAte splicing generate human soluble FLT1 variants and regulate the abundance of soluble FLT1 in the placenta." Faseb j **21**(14): 3885-3895.
Thomas, C. P., J. I. Andrews, N. S. Raikwar, E. A. Kelley, F. Herse, R. Dechend, T. G. Golos and K. Z. Liu (2009). "A Recently Evolved Novel Trophoblast-Enriched Secreted Form of fms-Like Tyrosine Kinase-1 Variant Is Up-Regulated in Hypoxia and Preeclampsia." The JouRNAl of Clinical Endocrinology & Metabolism **94**(7): 2524-2530.
Thomas, C. P., J. I. Andrews, N. S. Raikwar, E. A. Kelley, F. Herse, R. Dechend, T. G. Golos and K. Z. Liu (2009). "A recently evolved novel trophoblast-enriched secreted form of fms-like tyrosine kinase-1 variant is up-regulated in hypoxia and preeclampsia." J Clin Endocrinol Metab **94**(7): 2524-2530.
Torricelli, M., G. De Falco, P. Florio, M. Rossi, E. Leucci, P. Vigano, L. Leoncini and F. Petraglia (2007). "Secretory endometrium highly expresses urocortin messenger RNA and peptide: possible role in the decidualization process." Hum Reprod **22**(1): 92-96.
Torricelli, M., E. Ignacchiti, A. Giovannelli, A. Merola, E. Scarpetti, G. Calonaci, E. Picciolini, P. Florio, F. M. Reis, E. A. Linton and F. Petraglia (2006). "Maternalplasma corticotrophin-releasing factor and urocortin levels in post-term pregnancies." Eur J Endocrinol **154**(2): 281-285.
Toufaily, C., A. G. Lokossou, A. Vargas, É. Rassart and B. Barbeau (2015). "A CRE/AP-1-Like Motif Is Essential for Induced Syncytin-2 Expression and Fusion in Human Trophoblast-Like Model." PLOS ONE **10**(3): e0121468.
Trinh, X. B., W. A. A. Tjalma, P. B. Vermeulen, G. Van den Eynden, I. Van der Auwera, S. J. Van Laere, J. Helleman, E. M. J. J. Berns, L. Y. Dirix and P. A. van Dam (2009). "The VEGF pathway and the AKT/MTOR/p70S6K1 signalling pathway in human epithelial ovarian cancer." British JouRNAl of Cancer **100**(6): 971-978.
Tropper, P. J., W. B. Warren, S. M. Jozak, I. M. Conwell, R. I. Stark and R. S. Goland (1992). "Corticotropin releasing hormone concentrations in umbilical cord blood of preterm fetuses." J Dev Physiol **18**(2): 81-85.
Trudinger, B. J., W. B. Giles, C. M. Cook, J. Bombardieri and L. Collins (1985). "Fetal umbilical artery flow velocity waveforms and placental resistance: clinical significance." Br J Obstet Gynaecol **92**(1): 23-30.

Tsai-Morris, C. H., E. Buczko, Y. Geng, A. Gamboa-Pinto and M. L. Dufau (1996). "The genomic structure of the rat corticotropin releasing factor receptor. A member of the class II G protein-coupled receptors." J Biol Chem **271**(24): 14519-14525.
Tsatsanis, C., A. Androulidaki, T. Alissafi, I. Charalampopoulos, E. Dermitzaki, T. Roger, A. Gravanis and A. N. Margioris (2006). "Corticotropin-releasing factor and the urocortins induce the expression of TLR4 in macrophages via activation of the transcription factors PU.1 and AP-1." J Immunol **176**(3): 1869-1877.
Tsurumi, Y., T. Murohara, K. Krasinski, D. Chen, B. Witzenbichler, M. Kearney, T. Couffinhal and J. M. Isner (1997). "Reciprocal relation between VEGF and NO in the regulation of endothelial integrity." Nat Med **3**(8): 879-886.
Tu, H., A. J. Kastin and W. Pan (2007). "Corticotropin-releasing hormone receptor (CRHR)1 and CRHR2 are both trafficking and signaling receptors for urocortin." Mol Endocrinol **21**(3): 700-711.
Uckan, D., A. Steele, Cherry, B. Y. Wang, W. Chamizo, A. Koutsonikolis, E. Gilbert-Barness and R. A. Good (1997). "Trophoblasts express Fas ligand: a proposed mechanism for immune privilege in placenta and maternalinvasion." Mol Hum Reprod **3**(8): 655-662.
Ukah, U. V., J. A. Hutcheon, B. Payne, M. D. Haslam, M. Vatish, J. M. Ansermino, H. Brown, L. A. Magee and P. von Dadelszen (2017). "Placental Growth Factor as a Prognostic Tool in Women With Hypertensive Disorders of Pregnancy: A Systematic Review." Hypertension (Dallas, Tex. : 1979) **70**(6): 1228-1237.
Ulisse, S., A. Fabbri, J. C. Tinajero and M. L. Dufau (1990). "A novel mechanism of action of corticotropin releasing factor in rat Leydig cells." J Biol Chem **265**(4): 1964-1971.
Uzuki, M., H. Sasano, Y. Muramatsu, K. Totsune, K. Takahashi, Y. Oki, K. Iino and T. Sawai (2001). "Urocortin in the synovial tissue of patients with rheumatoid arthritis." Clin Sci (Lond) **100**(6): 577-589.
Vaillancourt, C., D. Lanoix, F. Le Bellego, G. Daoud and J. Lafond (2009). "Involvement of MAPK signalling in human villous trophoblast differentiation." Mini Rev Med Chem **9**(8): 962-973.
Valdenaire, O., T. Giller, V. Breu, J. Gottowik and G. Kilpatrick (1997). "A new functional isoform of the human CRF2 receptor for corticotropin-releasing factor." Biochim Biophys Acta **1352**(2): 129-132.
Vamvakopoulos, N. C. and G. P. Chrousos (1994). "Hormonal regulation of human corticotropin-releasing hormone gene expression: implications for the stress response and immune/inflammatory reaction." Endocr Rev **15**(4): 409-420.
Vamvakopoulos, N. C. and T. O. Sioutopoulou (1994). "Human corticotropin-releasing hormone receptor gene (CRHR) is located on the long arm of chromosome 17 (17q12-qter)." Chromosome Res **2**(6): 471-473.
Van de Velde, S., M. F. Hogan and M. Montminy (2011). "MTOR links incretin signaling to HIF induction in pancreatic beta cells." Proceedings of the National Academy of Sciences **108**(41): 16876-16882.
van den Brule, F., S. Berndt, N. Simon, C. Coulon, J. Le Goarant, C. Munaut, A. Noel, F. Frankenne and J. M. Foidart (2005). "Trophoblast invasion and placentation: molecular mechanisms and regulation." Chem Immunol Allergy **88**: 163-180.
Vanhaesebroeck, B., S. J. Leevers, G. Panayotou and M. D. Waterfield (1997). "Phosphoinositide 3-kinases: a conserved family of signal transducers." Trends Biochem Sci **22**(7): 267-272.
Vargas, A., C. Toufaily, F. LeBellego, E. Rassart, J. Lafond, B. Barbeau, A. Vargas, C. Toufaily, F. LeBellego, E. Rassart, J. Lafond and B. Barbeau (2011). "Reduced expression of both syncytin 1 and syncytin 2 correlates with severity of preeclampsia." Reproductive Sciences **18**: 1085-1091.

Vatish, M., H. S. Randeva and D. K. Grammatopoulos (2006). "Hormonal regulation of placental nitric oxide and pathogenesis of pre-eclampsia." Trends Mol Med **12**(5): 223-233.
Vatish, M., L. Tesfa, D. Grammatopoulos, E. Yamada, C. C. Bastie and J. E. Pessin (2012). "Inhibition of AKT activity and calcium channel function coordinately drive cell-cell fusion in the BEWO choriocarcinoma placental cell line." PLoS One **7**(1): e29353.
Vaughan, J., C. Donaldson, J. Bittencourt, M. H. Perrin, K. Lewis, S. Sutton, R. Chan, A. V. Turnbull, D. Lovejoy, C. Rivier, J. Rivier, P. E. Sawchenko and W. Vale (1995). "Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor." Nature **378**: 287.
Venihaki, M. and J. Majzoub (2002). "Lessons from CRH knockout mice." Neuropeptides **36**(2-3): 96-102.
Venihaki, M., S. Sakihara, S. Subramanian, P. Dikkes, S. C. Weninger, G. Liapakis, T. Graf and J. A. Majzoub (2004). "Urocortin III, a brain neuropeptide of the corticotropin-releasing hormone family: modulation by stress and attenuation of some anxiety-like behaviours." J Neuroendocrinol **16**(5): 411-422.
Verlohren, S., I. Herraiz, O. Lapaire, D. Schlembach, H. Zeisler, P. Calda, J. Sabria, F. Markfeld-Erol, A. Galindo, K. Schoofs, B. Denk and H. Stepan (2014). "New gestational phase-specific cutoff values for the use of the soluble fms-like tyrosine kinase-1/placental growth factor ratio as a diagnostic test for preeclampsia." Hypertension **63**(2): 346-352.
Vetter, D. E., C. Li, L. Zhao, A. Contarino, M. C. Liberman, G. W. Smith, Y. Marchuk, G. F. Koob, S. F. Heinemann, W. Vale and K. F. Lee (2002). "Urocortin-deficient mice show hearing impairment and increased anxiety-like behavior." Nat Genet **31**(4): 363-369.
Vianna, P., M. E. Bauer, D. Dornfeld and J. A. Chies (2011). "Distress conditions during pregnancy may lead to pre-eclampsia by increasing cortisol levels and altering lymphocyte sensitivity to glucocorticoids." Med Hypotheses **77**(2): 188-191.
Vicovac, L. and J. D. Aplin (1996). "Epithelial-mesenchymal transition during trophoblast differentiation." Acta Anat (Basel) **156**(3): 202-216.
Vita, N., P. Laurent, S. Lefort, P. Chalon, J. M. Lelias, M. Kaghad, G. Le Fur, D. Caput and P. Ferrara (1993). "Primary structure and functional expression of mouse pituitary and human brain corticotrophin releasing factor receptors." FEBS Lett **335**(1): 1-5.
Vitoratos, N., D. C. Papatheodorou, S. N. Kalantaridou and G. Mastorakos (2006). ""Reproductive" corticotropin-releasing hormone." Ann N Y Acad Sci **1092**: 310-318.
Wadhwa, P. D., J. F. Culhane, V. Rauh and S. S. Barve (2001). "Stress and preterm birth: neuroendocrine, immune/inflammatory, and vascular mechanisms." Matern Child Health J **5**(2): 119-125.
Wakahashi, S., K. Nakabayashi, N. Maruo, A. Yata, N. Ohara and T. Maruo (2008). "Effects of corticotropin-releasing hormone and stresscopin on vascular endothelial growth factor mRNA expression in cultured early human extravillous trophoblasts." Endocrine **33**(2): 144-151.
Wang, J., Y. Xu, Y. Xu, H. Zhu, R. Zhang, G. Zhang and S. Li (2008). "Urocortin's Inhibition of Tumor Growth and Angiogenesis in Hepatocellular Carcinoma via Corticotrophin-Releasing Factor Receptor 2." Cancer Investigation **26**(4): 359-368.
Wang, J., Y. Xu, Y. Xu, H. Zhu, R. Zhang, G. Zhang and S. Li (2008). "Urocortin's inhibition of tumor growth and angiogenesis in hepatocellular carcinoma via corticotrophin-releasing factor receptor 2." Cancer Invest **26**(4): 359-368.
Wang, L., F. Liu and M. L. Adamo (2001). "Cyclic AMP Inhibits Extracellular Signal-regulated Kinase and Phosphatidylinositol 3-Kinase/AKT Pathways by Inhibiting Rap1." JouRNAl of Biological Chemistry **276**(40): 37242-37249.

Warren, W. B., S. L. Patrick and R. S. Goland (1992). "Elevated maternalplasma corticotropin-releasing hormone levels in pregnancies complicated by preterm labor." Am J Obstet Gynecol **166**(4): 1198-1204; discussion 1204-1197.
Weel, I. C., V. R. Ribeiro, M. Romao, M. L. Matias, V. T. M Borges, J. C. Peracoli, J. P. Araujo Jr and M. T. S Peracoli (2015). "[62-OR]: Impaired autophagy in placenta of pregnant women with preeclampsia." Pregnancy Hypertension: An InteRNAtional JouRNAl of Women's Cardiovascular Health **5**(1): 32-33.
Wen, H. Y., S. Abbasi, R. E. Kellems and Y. Xia (2005). "MTOR: A placental growth signaling sensor." Placenta **26**: S63-S69.
Weninger, S. C., A. J. Dunn, L. J. Muglia, P. Dikkes, K. A. Miczek, A. H. Swiergiel, C. W. Berridge and J. A. Majzoub (1999). "Stress-induced behaviors require the corticotropin-releasing hormone (CRH) receptor, but not CRH." Proceedings of the National Academy of Sciences **96**(14): 8283-8288.
Wetzka, B., B. Sehringer, W. R. Schafer, S. Biller, C. Hor, E. Benedek, W. R. Deppert and H. P. Zahradnik (2003). "Expression patterns of CRH, CRH receptors, and CRH binding protein in human gestational tissue at term." Exp Clin Endocrinol Diabetes **111**(3): 154-161.
Wheeler, T., C. L. Elcock and F. W. Anthony (1995). "Angiogenesis and the placental environment." Placenta **16**(3): 289-296.
White, P. C., T. Mune and A. K. Agarwal (1997). "11 beta-Hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess." Endocr Rev **18**(1): 135-156.
Wice, B., D. Menton, H. Geuze and A. L. Schwartz (1990). "Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro." Exp Cell Res **186**(2): 306-316.
Wille, S., S. Sydow, M. R. Palchaudhuri, J. Spiess and F. M. Dautzenberg (1999). "Identification of amino acids in the N-terminal domain of corticotropin-releasing factor receptor 1 that are important determinants of high-affinity ligand binding." J Neurochem **72**(1): 388-395.
Wolff, N. C., R. M. McKay and J. Brugarolas (2014). "REDD1/DDIT4-independent MTORC1 inhibition and apoptosis by glucocorticoids in thymocytes." Mol Cancer Res **12**(6): 867-877.
Woods, R. J., A. Grossman, P. Saphier, K. Kennedy, E. Ur, D. Behan, E. Potter, W. Vale and P. J. Lowry (1994). "Association of human corticotropin-releasing hormone to its binding protein in blood may trigger clearance of the complex." J Clin Endocrinol Metab **78**(1): 73-76.
Woulfe, D., H. Jiang, A. Morgans, R. Monks, M. Birnbaum and L. F. Brass (2004). "Defects in secretion, aggregation, and thrombus formation in platelets from mice lacking AKT2." J Clin Invest **113**(3): 441-450.
Wullschleger, S., R. Loewith and M. N. Hall (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.
Xerri, L., E. Devilard, J. Hassoun, P. Haddad and F. Birg (1997). "Malignant and reactive cells from human lymphomas frequently express Fas ligand but display a different sensitivity to Fas-mediated apoptosis." Leukemia **11**(11): 1868-1877.
Xu, J., F. Xu, J. D. Hennebold, T. A. Molskness and R. L. Stouffer (2007). "Expression and role of the corticotropin-releasing hormone/urocortin-receptor-binding protein system in the primate corpus luteum during the menstrual cycle." Endocrinology **148**(11): 5385-5395.
Yamamoto, H., M. L. Flannery, S. Kupriyanov, J. Pearce, S. R. McKercher, G. W. Henkel, R. A. Maki, Z. Werb and R. G. Oshima (1998). "Defective trophoblast function in mice with a targeted mutation of Ets2." Genes Dev **12**(9): 1315-1326.
Yang, M., Z. M. Lei and V. Rao Ch (2003). "The central role of human chorionic gonadotropin in the formation of human placental syncytium." Endocrinology **144**(3): 1108-1120.

Yang, Z. Z., O. Tschopp, A. Baudry, B. Dummler, D. Hynx and B. A. Hemmings (2004). "Physiological functions of protein kinase B/AKT." Biochem Soc Trans **32**(Pt 2): 350-354.
Yang, Z. Z., O. Tschopp, M. Hemmings-Mieszczak, J. Feng, D. Brodbeck, E. Perentes and B. A. Hemmings (2003). "Protein kinase B alpha/AKT1 regulates placental development and fetal growth." J Biol Chem **278**(34): 32124-32131.
Yoon, D. J., C. Sklar and R. David (1988). "Presence of immunoreactive corticotropin-releasing factor in rat testis." Endocrinology **122**(2): 759-761.
Yung, H. W., S. Calabrese, D. Hynx, B. A. Hemmings, I. Cetin, D. S. Charnock-Jones and G. J. Burton (2008). "Evidence of placental translation inhibition and endoplasmic reticulum stress in the etiology of human intrauterine growth restriction." Am J Pathol **173**(2): 451-462.
Zachariades, E., H. Foster, A. Goumenou, P. Thomas, M. Rand-Weaver and E. Karteris (2011). "Expression of membrane and nuclear progesterone receptors in two human placental choriocarcinoma cell lines (JEG-3 and BEWO): Effects of syncytialization." Int J Mol Med **27**(6): 767-774.
Zeng, H., D. Zhao and D. Mukhopadhyay (2002). "FLT-1-mediated down-regulation of endothelial cell proliferation through pertussis toxin-sensitive G proteins, beta gamma subunits, small GTPase CDC42, and partly by Rac-1." J Biol Chem **277**(6): 4003-4009.
Zha, J., H. Harada, E. Yang, J. Jockel and S. J. Korsmeyer (1996). "Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L)." Cell **87**(4): 619-628.
Zhang, X., D. T. Odom, S.-H. Koo, M. D. Conkright, G. Canettieri, J. Best, H. Chen, R. Jenner, E. Herbolsheimer, E. Jacobsen, S. Kadam, J. R. Ecker, B. Emerson, J. B. Hogenesch, T. Unterman, R. A. Young and M. Montminy (2005). "Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues." Proceedings of the National Academy of Sciences of the United States of America **102**(12): 4459-4464.
Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold and J. M. Friedman (1994). "Positional cloning of the mouse obese gene and its human homologue." Nature **372**(6505): 425-432.
Zhao, L., C. J. Donaldson, G. W. Smith and W. W. Vale (1998). "The structures of the mouse and human urocortin genes (UCN and UCN)." Genomics **50**(1): 23-33.
Zhao, S., Y. Gu, R. Fan, L. J. Groome, D. Cooper and Y. Wang (2010). "Proteases and sFLT-1 release in the human placenta." Placenta **31**(6): 512-518.
Zhao, X. J., G. Hoheisel, J. Schauer and S. R. Bornstein (1997). "Corticotropin-releasing hormone-binding protein and its possible role in neuroendocrinological research." Horm Metab Res **29**(8): 373-378.
Zheng, J., I. M. Bird, A. N. Melsaether and R. R. Magness (1999). "Activation of the mitogen-activated protein kinase cascade is necessary but not sufficient for basic fibroblast growth factor- and epidermal growth factor-stimulated expression of endothelial nitric oxide synthase in ovine fetoplacental artery endothelial cells." Endocrinology **140**(3): 1399-1407.
Zhou, C. C., S. Ahmad, T. Mi, S. Abbasi, L. Xia, M. C. Day, S. M. Ramin, A. Ahmed, R. E. Kellems and Y. Xia (2008). "Autoantibody from women with preeclampsia induces soluble Fms-like tyrosine kinase-1 production via angiotensin type 1 receptor and calcineurin/nuclear factor of activated T-cells signaling." Hypertension **51**(4): 1010-1019.
Zhou, C. C., S. Ahmad, T. Mi, L. Xia, S. Abbasi, P. W. Hewett, C. Sun, A. Ahmed, R. E. Kellems and Y. Xia (2007). "Angiotensin II Induces Soluble fms-Like Tyrosine Kinase-1 Release via Calcineurin Signaling Pathway in Pregnancy." Circulation Research **100**(1): 88-95.
Zoumakis, E., A. N. Margioris, C. StouRNAras, E. Dermitzaki, E. Angelakis, A. Makrigiannakis, E. Koumantakis and A. Gravanis (2000). "Corticotrophin-releasing hormone

(CRH) interacts with inflammatory prostaglandins and interleukins and affects the decidualization of human endometrial stroma." Mol Hum Reprod **6**(4): 344-351.