Signalling Pathways of Oestrogen-Related Receptor Gamma (*ESRRG*) in Pregnancies complicated by Fetal Growth Restriction

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ABSTRACT

Scientific abstract of thesis entitled " Signalling Pathways of Oestrogen-Related Receptor Gamma (*ESRRG*) in Pregnancies complicated by Fetal Growth Restriction" submitted by Zhiyong Zou for the degree of PhD from the University of Manchester, 2022.

Fetal growth restriction (FGR), when a fetus does not achieve its growth potential, affects 10% of pregnancies in high-income countries. FGR is not only associated with increased risk of perinatal morbidity and mortality, but also increases the possibility of cardiovascular diseases or metabolic disorders in later life. To date, there is no effective method to prevent or treat FGR, except delivery of the infant. Understanding the underlying mechanisms responsible may help to develop therapies to prevent or treat the condition. FGR is associated with placental dysfunction, which has been attributed to various causes, including: hypoxic conditions in later pregnancy, prenatal exposure to the environmental contaminant BPA, and altered levels of some miRNAs. Reduced levels of oestrogen-related receptor gamma (*ESRRG*), a nuclear receptor highly expressed in the normal human placenta, is also observed in placentas complicated by FGR. Restoring *ESRRG* function may improve placental function, but the mechanisms regulating *ESRRG* signalling are currently not fully understood.

This thesis assessed whether hypoxia, BPA exposure and miRNAs modulate *ESRRG* signalling pathways, and explored whether this signalling pathway contributes to the abnormal cell turnover and hormone secretion that are features of FGR placentas. The programme of research had three aims:

1) To determine the role of *ESRRG* signalling pathways in FGR pregnancies and to assess whether hypoxia can regulate *ESRRG* expression and signalling in a cultured placental explant model. Placentas from FGR pregnancies and from pregnancies with appropriately grown infants were collected. ESRRG mRNA and protein expression

were decreased in FGR placentas; this was accompanied by a reduction in mRNA expression of several downstream genes. The impact of hypoxia on *ESRRG* was then determined using placental explants from term healthy placentas cultured in 1% O_2, 6% O_2, 21% O_2 or treated with cobalt chloride (200μM, $CoCl_2$) for four days of culture. The mRNA expression of *ESRRG* and its downstream genes were decreased in the explants cultured in 1% O_2 or treated with $CoCl_2$, with a reduced percentage of cells in cycle and an increase in apoptotic cells. Application of the *ESRRG* agonist, DY131, rescued abnormal cell turnover via restoring *ESRRG* signalling in the hypoxic placental explants.

2) To assess the impact of BPA on *ESRRG* signalling pathways, placental explants from 18 healthy term placentas (9 female and 9 male) were exposed to BPA (1pM-1μM) for up to 48 hours of culture. *ESRRG* mRNA levels were significantly increased in the explants treated with 1μM BPA for 24 hours. *ESRRG* signalling was sex-specific: down-regulation of ESRRG mRNA and protein expression in male placentas and up-regulation of ESRRG signals in female placentas was observed. BPA treatment did not alter hCG secretion, but induced LDH release from the female placentas with 1pM BPA exposure. Physiological BPA exposure did not alter the percentage of cells in cycle or apoptotic cells.

3) To establish if the *ESRRG* signalling pathway is modulated by miR-377. Normal term placental explants were treated with miR-377 mimics to overexpress miR-377. ESRRG mRNA and protein expression were significantly decreased in these explants, but there was no alteration in the expression of *ESRRG*'s downstream genes. miR-377 overexpression also reduced the numbers of cells in cycle and the percentage of apoptotic cells. miR-377 RNA expression was comparable between FGR and AGA placentas. The exact regulatory relationship between miR-377 and *ESRRG* is still unknown and whether miR-377 directly regulates *ESRRG* needs to be determined in future work.

Overall, placental *ESRRG* signalling is altered in FGR, and that *ESRRG* signalling is modulated by hypoxia and BPA, and potentially miR-377. This study also confirmed that BPA can regulate *ESRRG* signalling in a sex-specific manner in the villous explants model, which may explain why BPA exposure is predominantly linked to FGR in male neonates. Additionally, the results in this thesis have demonstrated, for the first time, that HIF-1 alpha acts as an upstream regulator to inhibit *ESRRG* signalling reducing the percentage of cells in cycle, and increasing the percentage of apoptotic cells in the cultured explants, which may contribute to the placental dysfunction underlying FGR pregnancies. Furthermore, DY131, an agonist of *ESRRG*, rescued the hypoxic damage observed in the explant model, providing the foundation for future research to investigate DY131 as a placental-specific therapy for FGR.

DECLARATION

I declare that no portion of the work has been submitted in support of an application for another degree or qualification.

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ABBREVIATIONS

µM	Micromoles
3'-UTR	3'-untranslated-region
4-OH	4-hydroxytamoxifen
AF	Activation function
AGA	Appropriate for gestational age
AGO	Argonaute
AR	Androgen receptor
BCA	Bicinchoninic Acid
BMI	Body mass index
BPA	Bisphenol A
BSA	Bovine serum albumin
CHIP	Chromatin immunoprecipitation
CK18	Cytokeratin 18
$CoCl_2$	Cobalt chloride
Ct	Threshold cycle
CYP	cytochrome P-450
CYP11B1	Cytochrome P450 family 11 subfamily B member 1
CYP11B2	Aldosterone synthase
CYP191.1	Cytochrome P-450
DAB	Chromogenic substrate diaminobenzidine
DBD	DNA-binding domain
DES	Diethylstilbestrol
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EFW	Estimated fetal weight
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Oestrogen receptor
ERRE	Oestrogen-related receptor elements
ERRs	Oestrogen-related receptors
ESRRA	Oestrogen-related receptor alpha
ESRRB	Oestrogen-related receptor beta
ESRRG	Oestrogen-related receptor gamma
EVT	Extravillous trophoblasts
FBS	Fetal bovine serum
FGR	Fetal growth restriction
GC-MS	Gas chromatography-mass spectrometry
GPCR30	G-protein-coupled receptor 30
hCG	Human chorionic gonadotrophin

HEK-293T cells	Human embryonic kidney 293 cells
HIF-1A	Hypoxia-inducible factor 1-alpha
HSD11B2	Hydroxysteroid 11-beta dehydrogenase
HSD17B1	Hydroxysteroid 17-beta dehydrogenase 1
HSDs	Hydroxysteroid dehydrogenases
HUVECs	Human umbilical vein endothelial cells
IBR	Individualised birth weight ratios
IHC	Immunochemistry
K^+ Channels	Potassium Channels
Klk1	Kallikrein 1
Klk6	Kallikrein 6
K_V7	Voltage-gated potassium
LBD	Ligand-binding domain
LDH	Lactate dehydrogenase
LRA	Luciferase reporter assay
MCAD	Medium-chain acyl-CoA dehydrogenase
miRISC	miRNA-induced silencing complex
miRNAs	microRNAs
MMP9	Metalloproteinase-9
mRNA	Messenger RNA
NaOH	Sodium hydroxide
NBF	Neutral phosphate-buffered formaldehyde solution
NR3B	Nuclear receptor 3B
NRF-1	Nuclear respiratory factor 1
NTD	N-terminal domain
O_2	Oxygen
p.c	Post conception
PBST	PBS-Tween-20
PDC	Pyruvate dehydrogenase complex
PDK4	Pyruvate dehydrogenase kinase 4
PE	Preeclampsia
PGC-1alpha	Proliferator-activated receptor coactivator 1-alpha
PLAC1	Placenta-specific protein 1
PPAR gamma	Peroxisome proliferator-activated receptor gamma
PR	Progesterone receptor
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RPLP0	60S acidic ribosomal protein P0

RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium-Dodecyl Sulphate polyacrylamide
sFlt-1	Soluble fms-like tyrosine kinase-1
SGA	Small for gestational age
siRNAs	Small interfering RNAs
SIRT1	Sirtuin 1
SMILE	Small heterodimer partner interacting leucine zipper protein
STB	Syncytiotrophoblast
TBST	TBS-Tween-20
TEXB	Total effective xenoestrogen burden
TNF-alpha	Tumor-necrosis factor alpha
U6	RNU6−1
UGT	UDP-glucuronosyltransferase
UPR	Unfolded protein response
VEGFA	Vascular endothelial growth factor A
WB	Western blot

1 CHAPTER 1: LITERATURE REVIEW

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1.1 Introduction

Placental dysfunction describes when the placenta fails to develop and/or function adequately to support the nutritional demands of the fetus, and is central to the development of both fetal growth restriction (FGR) and preeclampsia (Redman, 1991, Spinillo et al., 2019). FGR describes a fetus that does not reach its genetic growth potential. In clinical practice, this is often identified as a small for gestational age infant i.e. a baby whose estimated fetal weight (EFW) or birthweight is less than the 10th percentile for that stage of pregnancy (ACOG, 2019). However, being small for gestational age is not synonymous with FGR. True FGR affects 5%-10% of fetuses and is associated with both short and long-term complications including stillbirth, neonatal death, abnormal neurodevelopment, and cardiovascular and metabolic disorders in later life (Bernstein et al., 2000, Gardosi et al., 2005, Crispi et al., 2010, Ramirez-Velez et al., 2017, Pels et al., 2019). The majority of cases of FGR are mediated by abnormal placental structure and function (Spinillo et al., 2019). FGR may also co-exist with preeclampsia, which is defined as an elevation of maternal blood pressure with proteinuria occurring after 20 weeks' gestation (Brown et al., 2018). In addition to adverse effects on the fetus, preeclampsia is associated with maternal morbidity and mortality (Souza et al., 2013, Brown et al., 2018). Presently, there are no effective therapies to treat FGR or preeclampsia, leaving a decision between expectant management or delivery indicated by deterioration in fetal or maternal condition. Therapeutic advances are in part impaired by an incomplete understanding of the mechanisms underlying the placental dysfunction evident in FGR and preeclampsia. Therefore, the identification of key causal pathways amenable to therapeutic manipulation is an important goal for research in this area. Here we review the evidence for involvement of one such pathway, that of oestrogen-related receptor gamma (*ESRRG*) in the human placenta (Kumar and Mendelson, 2011).

Oestrogen-related receptor-gamma is a member of the ERR family of orphan nuclear receptors, which is highly expressed in the human placenta (Takeda et al., 2009). Evidence suggests that *ESRRG* serves an important role in trophoblast differentiation, proliferation, and invasion, and may be involved in blood pressure homeostasis (Luo et al., 2014, Zhu et al., 2018a). In addition, deficient expression of *ESRRG* is linked to impaired placental mitochondrial function (Poidatz et al., 2012), which could lead to inadequate energy supply and thus reduced energy expenditure within the placenta. Due to its wide range of functions in relevant biological processes, it is plausible that *ESRRG* may also play a role in placental dysfunction underlying pregnancies complicated with FGR or preeclampsia.

To consider whether the *ESRRG* pathway has a causal role in placental dysfunction, a review of the literature has been performed to: (i) summarize knowledge regarding the role of *ESRRG* in trophoblast, placental vascularisation and placental metabolism, (ii) discuss the evidence for aberrant expression of constituents of the *ESRRG* pathway in pregnancy complications, including FGR and preeclampsia, and (iii) consider the implications of altered *ESRRG* expression and how this may contribute to placental dysfunction.

1.2 Placental dysfunction underlying pregnancy complications

To assess whether a pathway may be involved in the pathophysiology of FGR and/or preeclampsia, its role in normal placental development requires consideration, followed by evaluation of whether the aberrant placental phenotype seen in these conditions is consistent with disruption of that pathway.

1.2.1 Normal placental development

Placental development begins as soon as the blastocyst implants around day 6 to 7 post conception (p.c.) (Kaufmann, 1995). In normal placental development, appropriate differentiation of cytotrophoblast cells, the trophoblast stem cell

population of the placenta, is important; two different pathways arise within the developing placental villus: the extravillous and villous lineages. As early as 5 gestational weeks (14 days after implantation), the extravillous trophoblasts (EVT) differentiate from cytotrophoblast cell columns and invade the uterus (interstitial invasion) and spiral arteries (endovascular invasion) to remodel the maternal blood vessels and ultimately produce dilated and compliant uterine arterioles (Figure 1.1A), thereby ensuring an adequate supply of oxygen and nutrients to support fetal growth (Pijnenborg et al., 1983, Kaufmann, 1995). Proliferation, differentiation and fusion of villous cytotrophoblast maintains the syncytiotrophoblast, which is syncytiotrophoblast renewal, the multinucleated outer layer of the placenta responsible for placental transport, protective and endocrine functions (Jones and Fox, 1991) (Figure 1.1A and 1.1B). The villous cytotrophoblast and syncytiotrophoblast, together with a core of villous stromal cells containing fetoplacental blood vessels, form the villous tree, which is the functional unit of the placenta (Jones and Fox, 1991) (Figure 1.1 A and 1.1B). There are five different types of villi, including mesenchymal villi, immature intermediate villi, stem villi, mature intermediate villi, and terminal villi. Terminal villi, which represent the final branches of the villous tree, exhibit a high degree of capillarization and fetoplacental vessels, are separated from maternal blood by a thin layer of syncytiotrophoblast and endothelial cells termed the vasculo-syncytial membrane, which is optimised for gas and nutrient exchange in human placenta (Kingdom et al., 2000). Consequently, there is a close relationship between terminal villous structure and function.

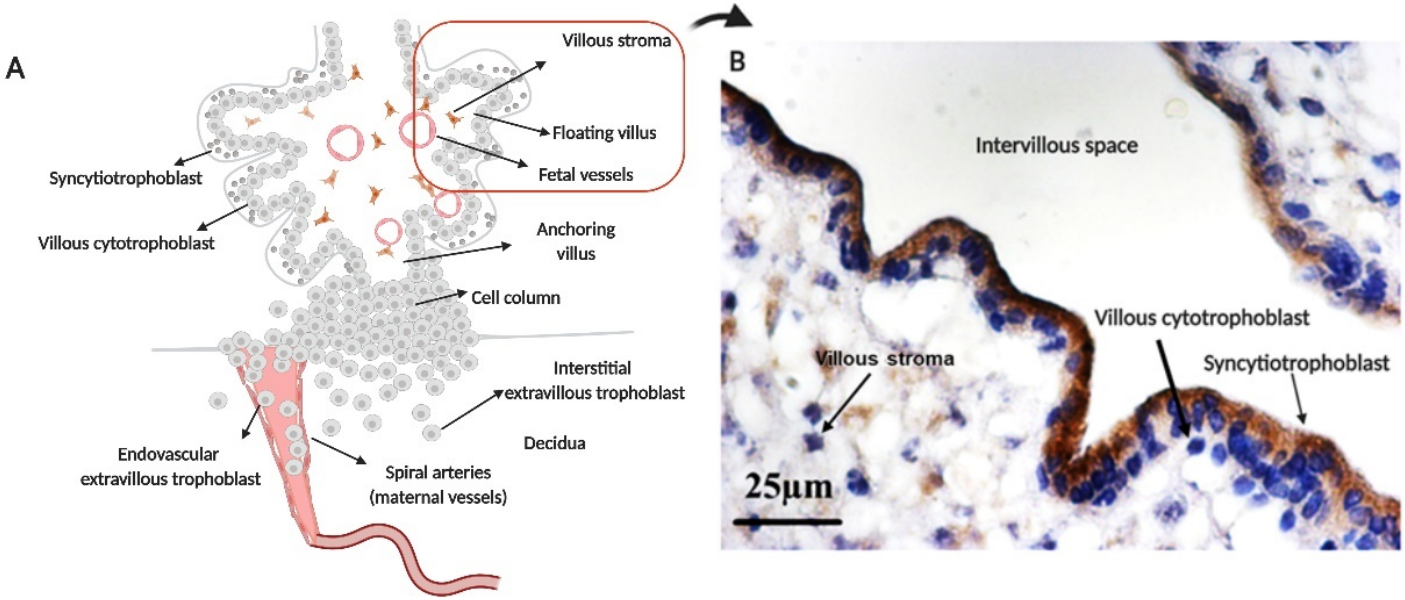

Figure 1.1 Schematic showing villous structure, trophoblast lineages and ESRRG localisation in the human placenta.

Figure 1.1A: Extravillous trophoblast situated at the end of the cell column invades the decidua and remodels the maternal spiral arterioles to produce dilated and compliant uterine vessels. Villous cytotrophoblast differentiates and fuses to form the outer multinucleated syncytiotrophoblast which transports nutrients and gases from the maternal to fetal circulation. Figure 1.1B is a micrograph showing immunostaining of ESRRG in first trimester placental explants. Localisation is mainly observed in the cytoplasm of the syncytiotrophoblast and cytotrophoblast. Arrows indicate villous cytotrophoblast, syncytiotrophoblast and villous stroma. Scale bar represents 25µm.

1.2.2 Endocrine activity in human placenta

The placenta is an endocrine organ and placental-related hormones are important for implantation, placentation, and vascular development. The multinucleated syncytiotrophoblast layer is most relevant for endocrine activity. The syncytiotrophoblast release human chorionic gonadotropin (hCG), progesterone, oestradiol, oestriol, human placental lactogen (HPL), and leptin and these hormones play an important role in placental development (Kliman et al., 1986, Bonduelle et al., 1988, Zhang et al., 2000, Pidoux et al., 2007, Costa, 2016). For example, human embryo is able to produce hCG before implantation and hCG also can promote the differentiation of the human cytotrophoblast into the syncytiotrophoblast *in vitro* (Bonduelle et al., 1988, Pidoux et al., 2007). Progesterone is a steroid hormone that is important to gestational maintenance and placental oestrogens included four different steroid hormones groups including oestrone (E1), 17β-oestradiol (E2), oestriol (E3) and oestetrol (E4). The placenta selectively secreting oestrogen and progesterone is also crucial for fetal growth by affecting other signalling pathways, such as matrix metalloproteinase 2 (MMP2), and MMP9 (Zhang et al., 2000, Bukovsky et al., 2003, Bjornstrom and Sjoberg, 2005).

1.2.3 Placental changes in FGR and preeclampsia

Compared to placentas from normal pregnancies, placentas from pregnancies complicated by FGR and/or pre-eclampsia may exhibit a number of structural and functional changes, including evidence of an unfolded protein response, increased trophoblast apoptosis and autophagy, and reduced trophoblast proliferation and metabolic function (Heazell et al., 2008, Heazell et al., 2011, Curtis et al., 2013, Burton and Jauniaux, 2018, Yung et al., 2019). In the syncytiotrophoblast, some nuclei are aggregated to form syncytial knots with features of apoptosis and disordered proliferation; the increased formation of syncytial knots is related to the conditions of placental dysfunction which have been found in FGR placentas (Macara et al., 1996, Heazell et al., 2007). FGR placentas also show decreased volume and surface area of terminal villi, with elongated and less-branched capillary loops (Jackson et al., 1995, Krebs et al., 1996). It is hypothesised that some of these changes in villous tissue are secondary to reduced invasion of extravillous trophoblast earlier in pregnancy, leading

to impaired perfusion of the intervillous space, and are characteristic of early onset preeclampsia and FGR (Redman et al., 2020). In late-onset pregnancy complications, normal placentation occurs, but impaired placental function arises in the later stages of pregnancy. This may be related to abnormal uterine capacity, with the induction of chorionic villous packing, compression of the intervillous space, and fetal hypoxia, which lead to “unexplained” stillbirths, late-onset fetal growth restriction, or late-onset preeclampsia (Redman et al., 2020). There may also be abnormalities of the fetal-placental vasculature, reduced nutrient transport capacity, and a reduction in placental weight, all of which combine to result in insufficient delivery of nutrients to the developing fetus (Roberts and Post, 2008). Insufficient placental functions and FGR pregnancies are also related to infection in prenatal, such as human cytomegalovirus (CMV) infection (Pereira et al., 2014).

The placenta is a metabolically active organ that consumes a large volume of oxygen throughout gestation, with energy provision mainly dependent on mitochondrial activity by glucose utilization (Diamant et al., 1975, Malek et al., 1996). An imbalance of placental mitochondrial function with excessive generation of reactive oxygen and nitrogen species in placentas is observed in pregnancy complications such as FGR, and preeclampsia (Atamer et al., 2005, Biri et al., 2007, Leduc et al., 2010). Taken together with the observation of altered perfusion of the intervillous space the critical relationship between hypoxia, reactive oxygen species (ROS), and how this leads to placental dysfunction needs to be considered.

1.3 A possible role of hypoxia / reactive oxygen species in placental dysfunction in FGR and Preeclampsia

As stated above, hypoxia and hypoxia-reoxygenation can contribute to the elevation of reactive oxygen species (ROS), which can lead to increased oxidative DNA damage and depletion of local antioxidant defenses (Hung and Burton, 2006, Kimura et al., 2013). Placental hypoxia has been reported in both FGR and preeclampsia (Kimura et al., 2013). Furthermore, a hypoxic environment can reproduce elements of the trophoblast phenotype seen in these conditions. Culture in 2% or 9% O_2 reduces differentiation and induces apoptosis in third trimester primary cytotrophoblast (Alsat

et al., 1996, Levy et al., 2000). Culture in 2% O_2 impaired differentiation and invasion in first-trimester primary cytotrophoblast (Genbacev et al., 1996), and term placental villous explants also exhibited reduced proliferation and induction of apoptosis when cultured at 1% compared to 6-8% O_2 (Heazell et al., 2008). Hypoxia (1% O_2) promotes stemness and differentiation in term cytotrophoblast by inhibiting GCM1 activity (Wang et al., 2022). Therefore, oxygen tension can modulate both the development of villous structure and trophoblast function. The molecular mechanisms responsible for these changes in trophoblast phenotype are still elusive, but recent reports suggest that it may, in part, be linked to the pathways induced by placental oxidative stress, such as the activation of an unfolded protein response (UPR) (Yung et al., 2008, Yung et al., 2019).

To understand the potential contribution of the *ESRRG* pathway in the pathogenesis of placental dysfunction underlying FGR and preeclampsia, the functions of *ESRRG* in pregnancy will be described, and the evidence that *ESRRG* signalling might be involved in the occurrence of placental dysfunction will be reviewed.

1.4 The ERR family

Oestrogen-related receptors (ERRs) are an NR3B (nuclear receptor 3B) group of the nuclear receptor subfamily, including ESRRA, ESRRB, and ESRRG, which are encoded by *ESRRA*, *ESRRB*, and *ESRRG* genes, respectively. The NR3B group of nuclear receptors is one of the larger NR3 classes and includes the hormone receptors for oestrogen, androgens, progesterone, aldosterone, and cortisol (Giguere et al., 1988, Giguere, 1999). Although ERRs share sequence homologies with the oestrogen receptor (ER), the transcription of ERRs is not activated by oestrogen, and information on the nature of endogenous ligands for ERRs remains to be established (Vanacker et al., 1999). ERRs can regulate transcription by binding to oestrogen-related receptor elements (ERRE) in target genes, which include several molecules involved in the cellular energy metabolic pathway (Giguere, 2008).

1.4.1 Structure of ERRs

According to their sequence homology and function, the structural features of ERRs include an activation function (AF)-1 domain /N-terminal domain (NTD), a DNA-

binding domain (DBD), a ligand-binding domain (LBD), and an AF-2 domain (Giguere, 1999) (Figure 1.2). The NTD is a non-conserved domain and it includes an AF-1 domain and a variable amino acid domain. In ESRRG and ESRRA, the NTD contains phosphorylation-dependent sumoylation sites that are embedded in a synergy control motif and may serve a role in regulating the transcriptional activity of ERRs (Tremblay et al., 2008). The synergy control motif may have a role in modulating higher-order interactions among transcriptional factors (Iniguez-Lluhi and Pearce, 2000). The ERRs' DBD has the highest sequence homology of the three ERR isoforms; ESRRB and ESRRA share 99% and 93% identical amino acid sequence with ESRRG respectively, which suggests that more than two ERRs might share some target genes (Heard et al., 2000). DBD contains two highly conserved zinc finger motifs, which recognize and bind oestrogen response element (ERE; 5'-AGGTCActgTGACCT-3') and a specific DNA sequence, denoted as an ERR response element (ERRE; 5'-TCAAGGTCA-3'). ERRs activate transcriptional activities as a monomer to recognize both ERE and ERRE, and the ERRs receptors dimerization including a homodimer, or a heterodimer, can modulate the transcriptional activities of ERRs (Johnston et al., 1997, Dufour et al., 2007). Moreover, ERRs and ERs have high homology in the DBD region (Giguere et al., 1988); 21% of ESRRA target promoters can be recognized by ESR1 in breast cancer cell lines (Deblois et al., 2009). Despite this, several genes can be regulated by both ESR1 and ERRs, including the human lactoferrin gene and monoamine oxidase B (Yang et al., 1996, Zhang et al., 2006b).

The final structural part of the ERRs is the LBD, a less conserved domain; there is a 77% sequence homology between the LBDs of ESRRB and ESRRG, and 61% homology between the ESRRA and ESRRG (Heard et al., 2000) (Figure 1.2). The homodimerization or heterodimerization of the LBD in ESRRG can also influence the transcriptional activities of ERRs; the homodimerization of ESRRG via the LBD can enhance the activity of transcription; conversely, heterodimerization with ESRRA inhibits the transcriptional activity of both receptors; this study suggests that mechanism for this repression is not due to the inability of ESRRG-ESRRA heterodimers binding to DNA (ERRE) and could result from inhibiting of common coactivators, like PGC-1alpha (Huppunen and Aarnisalo, 2004). The interaction between the LBD and its coactivator

is ligand-independent (Greschik et al., 2002). However, the crystal structure also shows that the LBD can interact with ligands by a flexible ligand-binding pocket and importantly from the perspective of understanding receptor signalling pathways, several synthetic molecules can inhibit or stimulate the transcriptional function of ERRs via the LBD, including proliferator-activated receptor coactivator 1-alpha, diethylstilbestrol (DES), and 4-hydroxytamoxifen (4-OH) (Tremblay et al., 2001a, Tremblay et al., 2001b, Kallen et al., 2004, Chao et al., 2006). Bisphenol A (BPA) is a chemical and environment contaminant used to produce plastics, which strongly binds to the ESRRG-LBD (Takeda et al., 2009). As the level of BPA in maternal blood and placental tissue is inversely related to fetal weight in human pregnancy (Troisi et al., 2014), BPA-mediated upregulation of placental ESRRG may provide a mechanistic link to explain the association between elevated BPA levels and FGR (Takayanagi et al., 2006, Okada et al., 2008). BPA responses in pregnancy may be sex-specific as previous studies observed maternal BPA exposure in humans correlated with FGR mainly in male offspring (Chou et al., 2011, Troisi et al., 2014), but the mechanism underlying these sex-differences are unclear.

Thus, the structure of the ERRs, specifically that of the LBD and DBD, is vital to the regulation of ERR signalling, including that of ESRRG. Furthermore, abnormal placental expression of ESRRG in FGR and preeclampsia suggests a potential role for ESRRG in the development or potentiation of these pregnancy complications (Luo et al., 2014, Zhu et al., 2018a). This thesis will consider how ESRRG is regulated, its effects in trophoblast and how this may contribute to the phenotypes of placental dysfunction observed in FGR and preeclampsia.

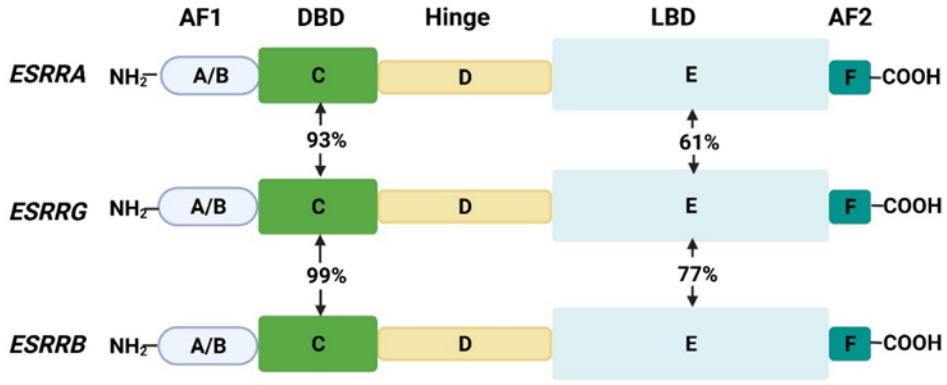

Figure 1.2 The structures of ERR family members

AF, activation function; DBD, DNA binding domain; LBD, ligand binding domain; *ESRRG*, oestrogen related receptor gamma; *ESRRB*, oestrogen related receptor beta; *ESRRA*, oestrogen related receptor alpha.

1.4.2 *ESRRG*

Both fetal and adult organs abundantly express ESRRG (Heard et al., 2000), including the placenta, heart, and brain (Heard et al., 2000, Takeda et al., 2009, Misra et al., 2017). ESRRG can regulate blood pressure homeostasis, due to the high expression of ESRRG in kidneys which mediate aldosterone-stimulated sodium and water reuptake (Alaynick et al., 2010). *Esrrg* null mice die during the first 72 hours of life with elevated serum potassium, and the genes that regulate serum potassium and blood pressure were decreased in the kidney in *Esrrg* knock-out mice; mRNA expression of the potassium channels, *Kcnj1*, *Kcne1*, and *Kcne2*, and kallikrein-kinin system genes kallikrein 1 (*Klk1*) and kallikrein 6 (*Klk6*), were significantly reduced in the kidneys of *Esrrg* null mice, (Alaynick et al., 2010). The body weight of *Esrrg* null mice was normal at E18.5, but decreased at day of birth (Alaynick et al., 2010). Other potential mechanisms by which *Esrrg* can regulate maternal blood pressure homeostasis during pregnancy are related to steroid 11beta-hydroxylase (*Cyp11b1*) and aldosterone synthase (*Cyp11b2*) (Luo et al., 2014). In *Esrrg* heterozygous (*Esrrg+/-*) pregnant mice, expression of *Cyp11b1* and *Cyp11b2* is decreased in the mouse adrenal cortex, resulting in reduced production of aldosterone and a reduction in blood pressure; conversely, expression of *Cyp11b1* and *Cyp11b2* in WT pregnant mice is increased after exposure to the *ESRRG* agonist DY131, which increased maternal blood pressure (Luo et al., 2014). Given that development of preeclampsia involves abnormal elevation of maternal blood pressure, dysregulation of *ESRRG* signaling in the kidney and adrenal cortex may contribute to this phenomenon.

Placental ESRRG expression also plays an important role in the maintenance of pregnancy. The placenta has the highest expression of ESRRG in the human reproductive system (Figure 1.1B) (Takeda et al., 2009); the mRNA and protein expression of ESRRG increases over gestation and the mRNA level of *ESRRG* is higher in primary villous cytotrophoblast compared to extravillous cytotrophoblast in first trimester placentas (Poidatz et al., 2012). ESRRG expression is dramatically increased during human cytotrophoblast cell differentiation, indicating a potential regulatory role (Kumar and Mendelson, 2011). Moreover, ESRRG also regulates genes involved in cellular energy homeostasis and metabolism; expression of key regulator genes

involved in mitochondrial biogenesis (*PGC-1A* and *NRF-1*) and energy metabolism (*PDK4* and *MCAD*) decreased after silencing *ESRRG* in human first trimester placental primary cytotrophoblast (Poidatz et al., 2012). As these studies indicate that *ESRRG* signaling may influence multiple aspects of normal placental function, we will review the evidence for the involvement of *ESRRG* in regulating trophoblast function, hypoxic responses, placental vascularisation, placental metabolism, and other regulators in the human placenta (Table 1.1 and Figure 1.3).

Table 1.1 Relationship of *ESRRG* to upstream regulators and downstream effectors demonstrated in studies of placenta.

Downstream effects	**Tissue/cell type/subject**	**Author**	**Study Methods**	**Main finding**	**Influence on trophoblast function**	**Definition of FGR / preeclampsia (where applicable)**
CYP191.1	Mid-trimester primary cytotrophoblasts	Kumar and Mendelson 2011	RT-PCR; WB; ChIP;	*ESRRG* is an oxygen-dependent transcription factor and mediates *CYP191.1* expression in trophoblast differentiation.	Differentiation	
CYP11B1	Late-onset PE placenta; Mouse model	Luo, et al. 2014	RT-PCR; IHC; WB; ChIP; LRA	*ESRRG* is increased in placenta in PE and can influence the blood pressure in pregnant mice by targeting *Cyp11b1*.	Reduced production of aldosterone	PE defined as maternal blood pressure (≥140/90 mmHg) and proteinuria (proteinuria ≥300mg per 24 hours or ≥1+ protein by dipstick from 2 random urine specimens or ≥2+protein by 1 dipstick) after 20 weeks of gestation.
Potassium (K^+) Channels	Mid-trimester primary trophoblast cells; Term FGR placenta	Luo, et al. 2013 Corcoran, et al. 2008	RT-PCR; WB; ChIP; LRA; Whole-genome	Hypoxia inhibits the expression of *ESRRG* and K^+ channels; *ESRRG* can regulate K^+ channels that may be associated with PE and	Differentiation	FGR defined as the individualized birth weight ratio (IBR) ≤ 5th centile for gestational age.

			gene expression arrays	identified three *ESRRG* potential effectors, including *HSD11B2*, *HSD17B1*, and *PLAC1*; Oxygen-sensitive K^+ channel gene $K_V9.3$ was increased in FGR placenta and $K_V2.1$ was increased in FGR placental vein.		
HSD11B2	Cytotrophoblast from mid-trimester placenta; Early-onset FGR placenta (n=15) and later-onset FGR placentas (n=4)	McTernan, et al. 2001 Luo, et al. 2013	RT-PCR	*HSD11B2* mRNA is decreased in *ESRRG* knockdown mid-trimester cytotrophoblasts. *HSD11B2* is decreased in both early-onset and later-onset FGR placentas, when compared with gestational matched normal placentas.	Not reported	FGR are diagnosed with at least three of four following ultrasound features: 1) fetal abdominal circumference ≤ 3rd centile for weeks of gestation, 2) abnormal fetal growth velocity 3) severe oligohydramnios (amniotic fluid index ≤3rd percentile for gestational age), 4) absent or reversed velocities in umbilical artery Doppler waveforms.

PLAC1	Placental villi of the human first trimester and term placenta Later-onset FGR placenta	Chang, et al. 2016 Sifakis, et al. 2018	RT-PCR; IHC; WB RT-PCR	*PLAC1* is increased during the trophoblast differentiation and low expression inhibits the cell fusion. *PLAC1* is increased in FGR placenta.	Differentiation Not reported	FGR were diagnosed as a fetus with reduced growth velocity, which is less than 10th centile after 20 gestational weeks.
HSD17B1	Late-onset FGR placenta; HTR-8 cell lines	Zhu, et al. 2018	RT-PCR; IHC; WB; LRA	*ESRRG* can regulate *HSD17B1* that is associated with FGR	Decrease invasion; proliferation	FGR defined as estimated fetal weight is less than 10th centile.
VEGFA	Mouse model	Luo, et al. 2014	RT-PCR	The expression of *VEGFA* is decreased in *ESRRG* deficient mice placenta	No detection reported	
PDK4	Primary trophoblast Bewo cell line	Poidatz, et al. 2012	RT-PCR, IHC	The expression of *ESRRG* is increased during trophoblast differentiation. *PDK4* is decreased after inhibiting *ESRRG* expression.	Differentiation	
MCAD	First trimester human primary cytotrophoblast;	Poidatz, et al. 2012	RT-PCR, IHC	*MCAD* is decreased after inhibiting *ESRRG* expression.	Differentiation	

	Bewo cell line					
Upstream						
BPA	Placentas from low birth weight infant; 587 children	Troisi, et al. 2014 Miao, et al. 2011	GC-MS analysis, Interview	Negative relationship between BPA and fetal weight; *ESRRG* is a receptor of BPA in the placenta.	Not reported	Low birth weight defined as the infant weight less than 2500g at birth;
PGC-1A	FGR and PE placenta; Late-onset FGR placenta	Poidatz, et al. 2015	IHC, RT-PCR, Quantification mitochondrial DNA	In FGR and PE placenta, the expression of *ESRRG*, *PGC-1A* and *SIRT1* is decreased.	No detection reported	FGR defined as a birth weight less than 10th centile. PE was diagnosed as an elevated maternal blood pressure (systolic and diastolic blood pressure ≥140/90mmHg) and proteinuria (≥ 300mg per 24 hours) after 20 weeks of gestation.
SIRT1	FGR and PE placenta;	Poidatz, et al. 2015	IHC, RT-PCR,	In FGR and PE placenta, the expression of *ESRRG*, *PGC-1A* and *SIRT1* is decreased.	No detection	FGR defined as a birth weight less than 10th centile.

	Late-onset FGR placenta		Quantification mitochondrial DNA			The definition of PE has been mentioned in the part of *PCG-1A*.
miR-320a	Late-onset PE placenta; HTR-8/SVneo	Gao, et al. 2018	RT-PCR; IHC; WB; LRA	miR-320a regulates *ESRRG* in PE	Decreased invasion; No change in proliferation and migration	PE defined as increased diastolic and systolic maternal blood pressure with proteinuria.
	HTR-8/SVneo; HUVECs	Liu, et al. 2018	RT-PCR; WB; LRA	miR-320a directly target *ESRRG* and may indirectly control the expression of *VEGFA* to influence the function of both trophoblast and vein endothelial cells.	Decreased invasion, proliferation and migration; Increase in apoptosis	
HIF-1A	Mid-trimester primary trophoblast cells	Kumar and Mendelson 2011	RT-PCR; WB; ChIP assay	*HIF-1A* can mediate *ESRRG* expression in trophoblast differentiation	Differentiation	

Abbreviations: *ESRRG*: oestrogen related receptor gamma; *CYP191.1*: cytochrome P-450; RT-PCR: reverse transcription polymerase chain reaction; WB: western blot; CHIP: chromatin immunoprecipitation; FGR: fetal growth restriction; *CYP11B1*: cytochrome P450 family 11 subfamily

B member 1; IHC: immunochemistry; LRA: luciferase reporter assay; PE: preeclampsia; *HSD11B2*: hydroxysteroid 11-beta dehydrogenase 2; *HSD17B1*: hydroxysteroid 17-beta dehydrogenase 1; *PLAC1*: placenta-specific protein 1; *VEGFA*: vascular endothelial growth factor A; *PDK4*: pyruvate dehydrogenase kinase 4; SGA: small for gestational age; PE: preeclampsia; *MCAD*: medium-chain acyl-CoA dehydrogenase; BPA: bisphenol A; GC-MS: gas chromatography-mass spectrometry; *PCG-1A*: peroxisome proliferator-activated receptor-gamma coactivator-1 alpha; HUVECs: human umbilical vein endothelial cells; *HIF-1A*: Hypoxia-inducible factor 1-alpha; *SIRT1*, sirtuin 1.

1.5 The effect of *ESRRG* on trophoblast function

1.5.1 Proliferation

Cell turnover defines as the constant shedding of dead cells with subsequent younger cell replacement. *ESRRG* knockdown reduces proliferation of the extravillous-like trophoblast cell line HTR-8/SVneo, by decreasing the expression of its downstream gene, 17beta-hydroxysteroid dehydrogenase type 1 (*HSD17B1*) (Zhu et al., 2018a). HSD17B1 is an enzyme capable of converting estrone to 17beta-estradiol in the metabolism of oestrogen. Abnormal expression of HSD17B1 has been reported in both FGR and preeclampsia (Zhu et al., 2018a); previous studies have revealed that a reduced plasma HSD17B1 expression level could be considered a potential prognostic factor for preeclampsia (Ohkuchi et al., 2012, Ishibashi et al., 2012). Ohkuchi *et al*. (Ohkuchi et al., 2012) examined 128 normal pregnant women and 30 pregnancies complicated with preeclampsia and found that reducing maternal plasma levels of HSD17B1 correlated with the occurrence of preeclampsia, implicating HSD17B1 in the pathogenesis of the disease, possibly by influencing the process of oestrogen metabolism. Since oestrogen can reduce the proliferation of HTR-8/SVneo cell line (Patel et al., 2015), this might suggest a relationship between low levels of *HSD17B1* in maternal serum, placental oestrogen metabolism, and trophoblast proliferation. Moreover, the mRNA and protein level of HSD17B1 was decreased in placentas complicated with FGR (Zhu et al., 2018a). Therefore, aberrant regulation of HSD17B1 by ESRRG may contribute to placental dysfunction, by its ability to regulate the proliferation of cytotrophoblast cells which is disrupted in FGR and preeclampsia.

1.5.2 Differentiation

The definition of differentiation is cytotrophoblast fused to form overlying syncytiotrophoblast. There is also evidence that ESRRG may influence cytotrophoblast differentiation via its role as a regulator of the aromatase CYP19A1, the voltage-gated potassium (K_V7) channel family, or via interactions with two other downstream genes, placenta specific protein-1 (*PLAC1*), and 11beta-hydroxysteroid dehydrogenase 2 (*HSD11B2*) (Kumar and Mendelson, 2011, Luo et al., 2013).

The cytochrome P-450 (CYP) family members include CYP11A1 and CYP19A1, and hydroxysteroid dehydrogenases (HSDs), such as 3beta-HSD and 17beta-HSD; these enzymes play a vital role in placental hormone synthesis and metabolism (Payne and Hales, 2004). C19 steroid precursors can be converted into oestrogen via activating aromatase P450, which is encoded by the *CYP19A1/ hCYP19* gene and only expressed in the syncytiotrophoblast, not in trophoblast stem cells or cytotrophoblast (Fournet-Dulguerov et al., 1987, Kamat et al., 1998). Notably, *ESRRG* has been shown to stimulate the expression of *hCYP19 in vitro*, via binding to its promotor to increase oestrogen levels in a 20% O_2 culture environment, which promotes trophoblast differentiation. When human second-trimester primary cytotrophoblasts were cultured in a hypoxic environment (2% O_2), both *ESRRG* and *hCYP19* expression decreased; however, elevating *ESRRG* expression restored *hCYP19* expression (Kumar and Mendelson, 2011).

ESRRG also induces mRNA and protein expression of the K_V7 family of potassium channels to regulate the differentiation of cytotrophoblast in second-trimester placentas. Voltage-gated K_V7 channels are encoded by the *KCNQ1-5* (alpha-subunit) and *KCNE1-5* (beta-subunit) genes. The human placenta expresses many potassium channel genes, including the *KCNQ* and *KCNE* families, and the expression of *KCNQ3* and *KCNE5* is markedly increased in placentas from pregnancies complicated with preeclampsia, particularly in the syncytiotrophoblast (Mistry et al., 2011). ESRRG induces mRNA and protein expression of the potassium channels KLK1, KCNQ1, KCNE1, KCNE3 and KCNE5 during primary cytotrophoblast differentiation, the effect of which was blocked by hypoxia (Luo et al., 2013). After examining the promotor, an ERRE located in the upstream region of the *KCNE1* and *KLK1* genes was identified, to which *ESRRG* can bind (Luo et al., 2013). In addition, expression of the oxygen-sensitive K^+ channel gene *$K_V9.3$* was increased in FGR placentas, and expression of *$K_V2.1$* was increased in chorionic plate veins from the same placentas (Corcoran et al., 2008). However, the relationship between *ESRRG* and expression of K^+ channels in functionally deficient placentas remains unclear.

There is also evidence that PLAC1 and HSD11B2 are downstream effectors of ESRRG, and these two genes can regulate cytotrophoblast differentiation (Luo et al., 2013).

The expression of PLAC1 is elevated during trophoblast differentiation and conversely, reduced expression of PLAC1 attenuates fusion of term primary human cytotrophoblast *in vitro* (Chang et al., 2016). Contrary to expectations, Sifakis *et al*. found high PLAC1 expression in FGR placentas at term (Sifakis et al., 2018), although this may be linked to the aberrant differentiation and trophoblast turnover reported in FGR (Heazell et al., 2011, Huppertz, 2011). Combined with the observations of Luo et al., these data suggest that the effect of PLAC1 on trophoblast differentiation might be mediated via ESRRG (Luo et al., 2013).

Another downstream gene of *ESRRG* is *HSD11B2*, an enzyme that converts active cortisol to inactive cortisone, which is expressed in villous syncytiotrophoblast (Pepe et al., 1999). Both mRNA and protein expression of HSD11B2 is induced during term primary cytotrophoblast differentiation, and it is considered a marker for trophoblast differentiation (Hardy and Yang, 2002, Homan et al., 2006). During pregnancy, HSD11B2 acts as a critical placental glucocorticoid barrier that protects the fetus from the harmful effects of excessive maternal glucocorticoids (Zhu et al., 2018b, Benediktsson et al., 1993). Placental HSD11B2 expression correlates with fetal weight and postnatal growth velocity (Benediktsson et al., 1993). McTernan *et al.* (McTernan et al., 2001) showed that placental HSD11B2 expression is decreased in FGR and demonstrated the importance of placental HSD11B2 in regulating fetal growth. Studies of SGA placentas also reported low HSD11B2 expression, which further revealed the relationship between HSD11B2 and fetal weight (Struwe et al., 2007). Placental HSD11B2 expression at birth is positively associated with fetal length at birth, whereas its expression is inversely related to growth velocity in the first year of life and might therefore be a predictor of postnatal growth of fetuses with FGR (Tzschoppe et al., 2009). These studies support the relationship between abnormal differentiation of cytotrophoblast seen in FGR and preeclampsia and expression of HSD11B2, although the roles of HSD11B2 in trophoblast function are still unclear. Since ESRRG regulates HSD11B2 (Luo et al., 2013), the reduced effect of HSD11B2 on trophoblast differentiation might be due to reduced levels of ESRRG in the presence of placental dysfunction.

1.5.3 Invasion

Invasion of the extravillous trophoblast into the uterine wall and subsequent remodeling of the uterine arterioles is critical for normal placental development and optimal uteroplacental perfusion. Knockdown of *ESRRG* resulted in deficient invasion of the extravillous-like HTR-8/SVneo cell line (Liu et al., 2018, Zhu et al., 2018a). Liu et al. showed that overexpression of microRNA (miR)-320a inhibited HTR-8/SVneo invasion by regulating ESRRG signalling (Liu et al., 2018). Furthermore, Zhu et al. demonstrated that reduced expression of ESRRG in HTR-8/SVneo cells significantly impaired invasion via regulation of HSD17B1 (Zhu et al., 2018a). Although a potentially significant finding, the relationship between *ESRRG* and the invasive capacity of extravillous trophoblast needs to be explored further using primary tissues.

1.5.4 The effect of *ESRRG* on placental vascularisation

Although the trophoblast is critically important to placental function, it is also widely acknowledged that impaired placental blood vessel development may be important in the aetiology of FGR (Hitschold et al., 1993). Several genes have been implicated in regulating placental vascularisation, including vascular endothelial growth factor A (*VEGFA*) (Ylikorkala et al., 2001, Burton et al., 2009). Maternal serum levels of VEGFA, an angiogenic factor that is crucial for placental angiogenesis during early gestation, are decreased in the 2nd and 3rd-trimester in pregnancies complicated with FGR (Bersinger and Odegard, 2005). Expression of VEGFA in primary vascular endothelial cells is also reduced in FGR placentas (Chui et al., 2014). The altered vascularisation seen in the placentas of women with FGR can potentially be attributed to dysregulated *ESRRG* expression. In mice, placentas from *Esrrg* -/- fetuses have significantly increased mRNA levels of *Vegfa,* compared with placentas from wild type fetuses (Luo et al., 2014), and in *Esrrg*+/- pregnant mice, circulating levels of the angiogenic, soluble receptor for *VEGF*, soluble fms-like tyrosine kinase-1(sFlt-1), were significantly reduced (Luo et al., 2014). In addition, direct regulatory interactions between *ESRRG* and *VEGFA* was confirmed in the other systems; in the mouse myoblast cell line C2C12, suppression of *Esrrg* can block the transcriptional expression of *Vegfa*, whilst in HEK-293T cells, *ESRRG* has been shown to activate the *VEGFA* promoter (Matsakas et al.,

2012). This indicates that ESRRG may affect placental vascularisation via its regulation of VEGFA.

The link between ESRRG and the development of placental vessels also could be associated with potassium channel expression or BPA exposure. One of the potential downstream effectors of *ESRRG*, potassium channels, play a role in placental vascularization, which may be mediated by *ESRRG* (Wareing et al., 2006, Wareing, 2014). In addition, an upstream regulator of *ESRRG*, BPA, altered the vascularisation of mice placentas. Exposure to BPA in pregnancy increased the total area of the maternal blood spaces, and embryonic capillaries in the labyrinth layer, which may be mediated through *ESRRG* signalling pathways (Tait et al., 2015). These studies all suggest that *ESRRG* may be involved in regulating placental vascularisation, however further studies are required in humans to confirm this.

1.5.5 *ESRRG* and placental metabolism

There is accumulating evidence that *ESRRG* plays a role in the regulation of several mitochondrial functions, including mitochondrial biogenesis, oxidative phosphorylation, and fatty acid oxidation, in the heart, kidney, skeletal muscle, and placenta (Huss et al., 2002, Alaynick et al., 2007, Dufour et al., 2007, Kubo et al., 2009, Alaynick et al., 2010, Fan et al., 2018). In the human placenta, *ESRRG* regulates mitochondrial function in the villous trophoblast by controlling gene networks involved in mitochondrial biogenesis and fat and glucose metabolism, including pyruvate dehydrogenase kinase 4 (*PDK4*), medium chain acyl-CoA dehydrogenase (*MCAD*), sirtuin 1 (*SIRT1*) and peroxisome proliferator-activated receptor gamma (PPAR gamma) coactivator 1 alpha (*PGC-1A*) (Poidatz et al., 2012).

In human term primary villous cytotrophoblast, expression of *PDK4* and *MCAD* was decreased after knockdown of *ESRRG* expression (Poidatz et al., 2012), which implicates *ESRRG* as a potential regulator of placental fatty acid oxidation and glucose metabolism mediated via these genes. PDK4 can phosphorylate the pyruvate dehydrogenase complex (PDC), which facilitates the conversion of pyruvate to acetyl-CoA in mitochondria, to inhibit the activity of PDC (Sugden and Holness, 2003); MCAD is an enzyme which catalyzes the initial step of mitochondrial fatty acid oxidation (FAO)

(Schulz, 1991). ESRRG can stimulate the expression of PDK4 in human liver cell lines (HepG2 cells) and rat hepatoma cells (Zhang et al., 2006a, Lee et al., 2012); regulation of the promoter of *PDK4* by *ESRRG* has been observed in mammary epithelial cells by using both ChIP and luciferase reporter assays, and the activation of the *ESRRG-PDK4* pathway attenuates glucose oxidation and decrease cell death and apoptosis (Kamarajugadda et al., 2012). Thus, a reduction in *ESRRG* would be expected to be associated with increased apoptosis and cell death, as is observed in FGR. This suggests that the *ESRRG-PDK4* signalling pathway in human placentas might contribute to placental dysfunction. *MCAD* is one of the targets of *ESRRA* and the increased expression of the *ESRRA-MCAD* pathway serves an important role in the decidualization of human primary endometrial stromal cells (Bombail et al., 2010). Since *ESRRG* is structurally similar to *ESRRA*, it is possible that *ESRRG* also contributes to the regulation of *MCAD*, however further studies are needed to investigate this and to determine the role of the *ESRRG-PDK4* pathway in placental dysfunction.

SIRT1, *PGC-1A,* and *PGC-1B* are also coactivators of *ESRRG* that have known roles in regulating placental metabolism. SIRT1, a NAD(+)-dependent protein deacetylase, is expressed ubiquitously in different organs and is required for many cellular processes related to differentiation and metabolism (Leibiger and Berggren, 2006). SIRT1 is expressed in both the syncytiotrophoblast and cytotrophoblast (Lappas et al., 2011). Findings from two recent studies in mice indicate that SIRT1 plays a key role in trophoblast differentiation and placental development (McBurney et al., 2003, Arul Nambi Rajan et al., 2018). The differentiation of mouse trophoblast stem cells obtained from *Sirt1*-null mice was blunted *in vitro* (Arul Nambi Rajan et al., 2018), resulting in fetuses with FGR, and smaller placentas with deficient morphology including a thickened chorion and a more hypercellular labyrinth were observed (McBurney et al., 2003, Arul Nambi Rajan et al., 2018). Wilson et al. found SIRT1 can deacetylate and interact with ESRRA *in vivo* and *in vitro*, which also suggests a potential interaction between ESRRG and SIRT1 (Wilson et al., 2010), since *ESRRA* and *ESRRG* have structural and functional similarities. In HepG2 cells, small heterodimer partner interacting leucine zipper protein (*SMILE*) expression and its ability to repress ESRRG transactivation and downstream signaling, is dependent on the expression of

SIRT1 (Xie et al., 2009). SIRT1 can also positively regulate the expression of another ESRRG coactivator, PGC-1alpha (Gerhart-Hines et al., 2007, Amat et al., 2009). PGC-1alpha and its family member, PGC-1beta, act as transcriptional co-regulators of ESRRA and ESRRG to influence metabolism in many diseases, such as cardiovascular disease and cancer (Huss et al., 2002, Liu et al., 2005, Torrano et al., 2016, Luo et al., 2017). In human placental tissue, the mRNA expression level of *PGC-1A* and *SIRT1* correlated with that of *ESRRG* in pregnancies complicated with preeclampsia and FGR (Poidatz et al., 2015a); low mRNA levels of *ESRRG*, *PGC-1A* and *SIRT1* have all been reported in FGR placentas (Poidatz et al., 2015a). Together, these studies suggest that methods to modulate both *ESRRG* and its transcriptional co-regulators, may provide a potential therapeutic strategy to improve placental metabolism and fetal growth. In addition, this thesis will review the microRNAs (miRNAs) that have been identified as upstream regulators of *ESRRG,* and which may also contribute to the etiology of placental dysfunction.

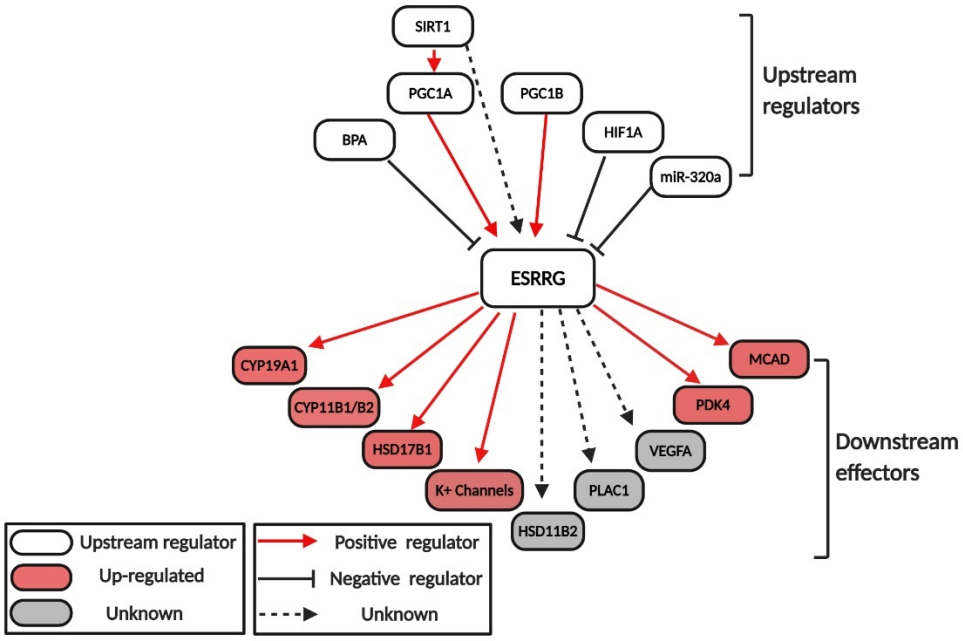

Figure 1.3 Diagrammatic representation of known upstream regulators and downstream effectors of ESRRG in the placenta.

Expression of the depicted genes is known to be altered in the FGR or preeclampsia placenta, where a red arrow indicates a positive effect and a black line indicates a negative effect. Meanwhile, a black dot arrow suggests an unclear regulation between *ESRRG* and downstream effectors. *CYP11B1*: cytochrome P450 family 11 subfamily B member 1; *HSD11B2*: hydroxysteroid 11-beta dehydrogenase 2; *HSD17B1*: hydroxysteroid 17-beta dehydrogenase 1; *PLAC1*: placenta-specific protein 1; *VEGFA*: vascular endothelial growth factor A; *PDK4*: pyruvate dehydrogenase kinase 4; *MCAD*: medium-chain acyl-CoA dehydrogenase; BPA: bisphenol A; PCG-1 alpha/beta: peroxisome proliferator-activated receptor-gamma coactivator-1 alpha/beta; HIF-1 alpha: Hypoxia-inducible factor 1-alpha. Image created with BioRender.com.

1.5.6 Regulation of *ESRRG* in the human placenta by miRNAs

miRNAs are short non-coding RNAs with 19-23 nucleotides which post-translationally reduce gene expression in both animals and plants by mediating argonaute (AGO) binding to the 3'-untranslated-region (3'-UTR) of mRNA (Baek et al., 2008). Base pairing of 2-7 nucleotides of miRNAs targets on mRNA 3'-UTR (Lewis et al., 2005, Grimson et al., 2007). The miRNA-induced silencing complex (miRISC), which includes the miRNAs and AGO, degrades target mRNA and represses protein translation. Different miRNAs are expressed in specific tissues, and by regulating different sets of target genes, specific miRNAs can mediate many cellular processes, such as differentiation, proliferation, and invasion (Anton et al., 2013, Li et al., 2014). The diversity of mRNA 3'-UTR targeted by miRNAs influences miRNA-mediated regulation, including exon skipping (Han et al., 2018). In humans, more than 60% of protein-coding genes are thought to be regulated by miRNAs, many of which are specifically expressed in the placenta (Friedman et al., 2009). Expression of numerous miRNAs is altered in pregnancy complications such as FGR and preeclampsia, which are associated with placental dysfunction (Friedman et al., 2009, Zhang et al., 2010, Hromadnikova et al., 2015b). The following miRNAs are associated with placental dysfunction and have been identified as potential upstream regulators of *ESRRG*.

1.5.6.1 miR-320a

miR-320a levels are increased in the placentas of women with late-onset preeclampsia, and overexpression of miR-320a in HTR-8/SVneo cells inhibits mRNA and protein expression of ESRRG (Gao et al., 2018). Key functional roles for *ESRRG* in the placenta appear to be modulated by miR-320a; direct regulation of *ESRRG* by miR-320a inhibits migration, invasion, and proliferation and indirectly modulates levels of *VEGFA* in both HTR-8/SVneo cells and human umbilical vein endothelial cells (HUVECs) (Gao et al., 2018, Liu et al., 2018). However, to our knowledge, expression levels of miR-320a in FGR placentas has yet to be assessed.

1.5.6.2 Other *ESRRG* regulatory miRNAs

Several other miRNAs have been implicated in placental dysfunction by regulating proliferation, invasion, or invasion of trophoblastic-like cell lines, and by reducing

ESRRG expression in other cell lines, these include miR-378a-5p, miR-424, miR-377, and miR-204-5p (Eichner et al., 2010, Cheng et al., 2018, Zou et al., 2019). miR-378a-5p inhibits both mRNA and protein levels of ESRRG in the breast cancer cell line, BT-474 (Eichner et al., 2010); it also enhances the invasion and migration of HTR8/SVneo cells and reduces BeWo cell differentiation (Luo et al., 2012, Nadeem et al., 2014). miR-424 expression was increased in FGR placentas (Huang et al., 2013) and miR-424 overexpression inhibited protein expression of ESRRG in HTR/8SVneo cells (Zou et al., 2019). However, this study did not identify a regulatory relationship between miR-424 and the 3'-UTR of *ESRRG,* thus more in-depth studies of miR-424 are required in the future (Zou et al., 2019).

miR-377 is more highly expressed in human term placentas than first-trimester placentas. Overexpression of miR-377 in first-trimester placental explants reduced cytotrophoblast proliferation (Farrokhnia et al., 2014) and miR-377 inhibits the expression levels of *SIRT-1* in human retinal endothelial cells. Taking into account the reported interaction between *ESRRG* and *SIRT-1* in the human placenta, miR-377 may also regulate ESRRG expression (Cui et al., 2019). As overexpression of miR-204-5p reduced the invasion of BeWo cells and JEG3 cells (Yu et al., 2015), and miR-204-5p overexpression reduced the differentiation of C1C12 myoblast cells by directly targeting 3'-UTR of *ESRRG*, there may be a direct regulatory relationship between miR-204-5p and *ESRRG* in the placenta (Cheng et al., 2018). Since the above studies only used cell lines to assess trophoblast function, more data derived from primary placental models are needed, specifically those that focus on the relationship between individual miRNAs, *ESRRG* and its downstream effectors, and their roles in the etiology of FGR; these relationships have been summarized in Figure 1.3.

Manipulation of the expression of miRNAs upstream of *ESRRG* may represent an additional approach to correct placental dysfunction; accumulating studies *in vivo* and *in vitro* indicate the possibility of developing an inverse agonist of *ESRRG* as a promising treatment for *ESRRG*-related anaplastic thyroid cancer, breast cancer, and type 2 diabetes (Kim et al., 2012, Kim et al., 2019b, Vernier et al., 2020). An inverse agonist defines as a ligand that binds to the same receptor-binding site as an agonist which produce an opposite response as an agonist. ESRRG inverse agonist, GSK-5182,

inhibits ESRRG transactivation by enhancing the interaction between ESRRG and its repressor, small heterodimer partner interacting leucine zipper protein, (SMILE) (Xie et al., 2009). Our group has demonstrated that targeted miRNA inhibitors can be used to selectively manipulate placental function: targeted inhibition of trophoblast miR-145 and miR-675 expression promoted cytotrophoblast proliferation in human first-trimester villous placental explants and increased fetal and placental weight when administered intravenously to pregnant mice (Beards et al., 2017). Therefore, exploring the regulatory pathway of *ESRRG* in the human placenta could inform the development of potential new therapeutic approaches for pregnancy complications involving placental dysfunction, like FGR or preeclampsia.

1.5.7 The effect of *ESRRG* in response to hypoxia

A hypoxic environment alters the expression of many genes which are associated with trophoblast differentiation. The most well-studied oxygen sensor in trophoblast is hypoxia-inducible factor 1 alpha (*HIF1A*), which is reported to be elevated in FGR and pre-eclampsia (Rajakumar et al., 2004, Robb et al., 2017). HIF-1alpha regulates *ESRRG* expression in human trophoblast: culture in a 2% O_2 environment activates *HIF-1A* and decreases the expression of *ESRRG* and *hCYP19* (Kumar and Mendelson, 2011). Conversely, knockdown of *HIF-1A* in trophoblast prevents *ESRRG* suppression under hypoxic conditions (Kumar and Mendelson, 2011). Collectively, these findings demonstrate that *ESRRG* serves as an oxygen-dependent transcriptional factor regulated by *HIF-1A* to control the expression of downstream *hCYP19*. This relationship appears to be maintained *in vivo*, as low ESRRG expression has been reported in placentas from FGR pregnancies, which often show evidence of hypoxia and/or oxidative stress (Takagi et al., 2004). A preliminary study in a south Chinese population examined the mRNA and protein level of ESRRG in 28 FGR placentas and 30 matched appropriate for gestational age (AGA) placentas, and reported lower expression of *ESRRG* in FGR placentas (Zhu et al., 2018a). Poidatz et al. (Poidatz et al., 2015a) also reported lower mRNA expression of *ESRRG* in 39 FGR placentas compared with 30 controls in a European population. These studies support the hypothesis that *ESRRG* might play a role in placental dysfunction originating from placental hypoxia.

1.5.8 *In vitro* model of placentas

There are some *in vitro* models which have been developed to study different aspects of placental functions, including cells from gestational choriocarcinoma, immortalized trophoblasts, or isolated primary cytotrophoblast, placental villous explants, organoids, and placenta-on-a-chip (Okae et al., 2018, Rossi et al., 2018, Corro et al., 2020). Trophoblastic cell lines, like BeWo cells or JEG-3 cells, are easy to get and culture and they have many characteristics of primary cytotrophoblast, like hCG secretion, but these cell lines are derived from trophoblast choriocarcinoma, and they behave different to physiological cells (Xu et al., 2019, Narciso et al., 2019). Also, these cell lines are not suitable to study different stages of pregnancies. Although isolated primary cytotrophoblast is a physiological cell model and is suitable to explore cytotrophoblast function in different stages of pregnancies, this is hampered by the limited amount of primary trophoblasts, contamination with other placental cell types, and rapid ceased proliferation. Human trophoblast stem cells (hTSCs) were established in 2018 and these cells can produce three major trophoblast lineages (Okae et al., 2018). It has been found that the transcriptomes and methylomes are similar between human trophoblast stem cells and primary trophoblast. And the trophoblast stem cells have been used to mimic trophoblast invasion during implantation in mice (Okae et al., 2018). However, the process to generate stem cells is inefficient and also money-consuming. All these two-dimensional-(2D) cells model lack the complexity of the human placenta with multiple layers and cell types. 3D organoid models provide important advantages by allowing specific cell-cell interactions and organoids can be patient-specific. But organoids mimic some, not all structure and function of the real organ as they are lacking vasculature and may lack key cell types *in vivo*, and some organoids only replicate the early stage of organ development (Rossi et al., 2018, Corro et al., 2020). An organ-on-chip is an artificial model of the placenta on the cell culture chip, which can maintain *in vivo* architecture, and reconstitute the structural, microenvironmental, and function complexity of a human placenta, but most organs-on-chips lack vasculature and also are difficult to adapt to high-throughput screening (Lee et al., 2016). Villous explants, a three-dimensional model, have been widely used to explore nutrient transport, metabolism,

endocrine function, and cell function in the placenta (Heazell et al., 2008, Renshall et al., 2021). Placental villous explants have the advantage that they are composed of mixed cell types and retained cell relationships *in vivo* tissue. Compared to organoid and organs-on-chips, villous explants are economical. Another advantage is this model can be used to study placental sex-specific differences. The disadvantage of villous explants is the unknown effect of specific cell types due to the complexity of the explant model.

1.6 Summary

Even though many studies have focused on the pathogenesis of placental dysfunction underlying FGR and preeclampsia, the precise pathophysiological mechanisms and biochemical pathways in the placenta are still unclear, which limits options for therapeutic discovery, making a better understanding of the underpinning placental pathways a priority. The most obvious changes in the placenta in FGR and preeclampsia include abnormal trophoblast function, increased cell death, altered metabolism and nutrient transport, hypoxia and oxidative stress, and aberrant villous structure. Since *ESRRG* is highly expressed in the human normal term placenta, and it holds key roles in the regulation of cell invasion, differentiation, cellular energy homeostasis, hypoxic responses and metabolism, we argue that involvement of the *ESRRG* pathway in the placental dysfunction underlying FGR and preeclampsia is plausible, and thorough exploration may offer new therapeutic options. In support of this hypothesis, several studies have revealed significantly lower levels of ESRRG mRNA and protein in the human placenta in FGR, and *ESRRG* can regulate the invasion and proliferation of human trophoblast cell lines. Furthermore, additional evidence of disruption of both upstream regulators and downstream effectors of *ESRRG* provides evidence that the pathway is intact, and functions as expected in the human placenta. These data highlight that *ESRRG* may be involved in the development and pathogenesis of placental dysfunction by influencing trophoblast function and further studies of the regulation of this pathway are needed. By better understanding the intrinsic role of *ESRRG* as a regulator of trophoblast function, metabolism, and cell turnover, this in turn might provide new ideas for the treatment of placental dysfunction underpinning FGR and preeclampsia in the future.

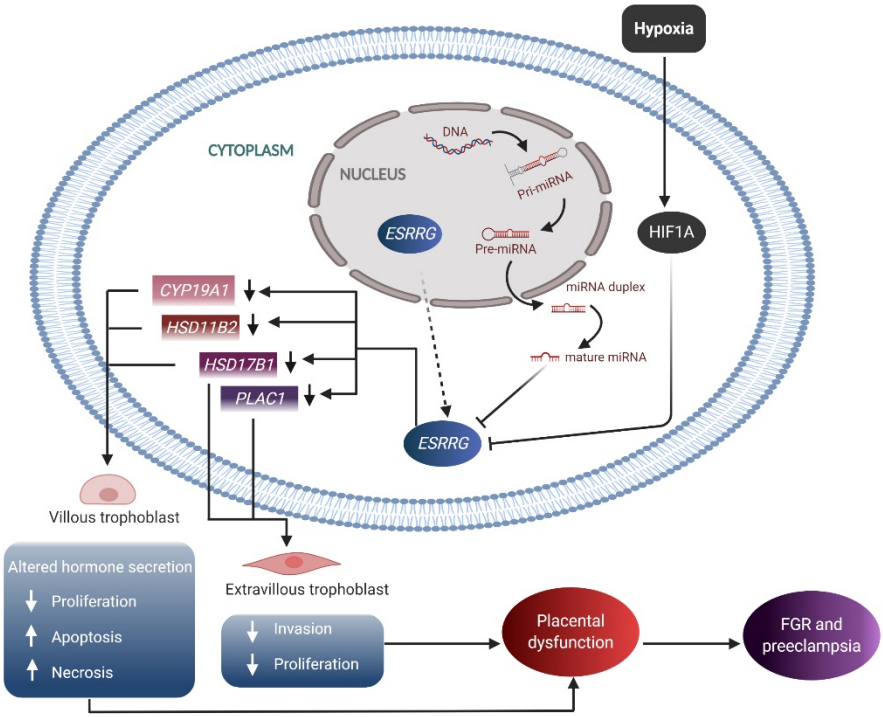

Figure 1.4 Proposed mechanism by which placental *ESRRG* expression and function is altered in pregnancies complicated by FGR and preeclampsia.

The hypoxic environment of the maternofetal interface in FGR results in upregulation and activation of HIF1alpha in the placenta, resulting in inhibition of *ESRRG* expression. The expression of several miRNAs is upregulated in FGR and we propose that amongst these are key *ESRRG* regulatory miRNAs. Binding of these miRNAs to the 3'UTR of *ESRRG* results in mRNA degradation and inhibition of ESRRG protein translation. This leads to reduced expression of genes downstream of *ESRRG*, including *CYP191.1*, *HSD17B1*, *HSD11B2* and *PLAC1*. These downstream genes play an important role in placental hormone production and regulating different aspects of cytotrophoblast function, including villous cytotrophoblast proliferation and extravillous trophoblast invasion. Image created with BioRender.com.

1.7 Hypothesis and Aim

The hypothesis of this study is that *ESRRG* expression is reduced in placentas in pregnancies complicated with FGR and this abnormal expression is related to the observed placental dysfunction (Figure 1.4). This thesis will address the overall proposal that (i) *ESRRG* is involved in regulation of, trophoblast turnover, and (ii) dysregulation of *ESRRG* signaling, induced by hypoxia, exposure to BPA or changes in the expression or activity of upstream miRNAs, could contribute to placental dysfunction, and could be one of the causes of the abnormal STB turnover and hormone secretion that is a feature of FGR.

Hypothesis 1:

Placental hypoxia, which is a feature of FGR, alters *ESRRG* signalling, leading to changes in trophoblast cell turnover, hCG secretion and LDH release.

Aim 1: To assess the expression of *ESRRG* and its downstream genes in placentas from FGR and AGA pregnancies.

Aim 2: To investigate whether hypoxia modulates trophoblast integrity, cell turnover and hCG secretion via an effect on the *ESRRG* signalling.

Aim 3: To explore whether DY131, an agonist of *ESRRG*, can restore the aberrant *ESRRG* signalling and trophoblast turnover and differentiation induced by hypoxia.

Hypothesis 2:

Exposure of human placental tissue to BPA alters *ESRRG* expression and placental function in a sex-specific manner.

Aim 1: To assess the expression of *ESRRG* and its downstream genes in cultured villous explants exposed to BPA.

Aim 2: To explore whether BPA exposure modulates the number of proliferating and/or apoptotic cells, trophoblast integrity and trophoblast differentiation in cultured villous explants.

Hypothesis 3:

Changes in the expression of miRNAs that reduce *ESRRG* signaling and alter one or more aspects of trophoblast function.

Aim 1: To determine the levels of miRNAs which may regulate *ESRRG* in placentas from FGR and AGA pregnancies.

Aim 2: To investigate whether miR-377 can regulate *ESRRG* signalling in cultured villous explants.

Aim 3: To describe whether the miR-377-*ESRRG* signalling pathway regulates trophoblast integrity, trophoblast differentiation and cell turnover in cultured villous explants.

2 CHAPTER 2: MATERIALS AND METHODS

Unless specifically mentioned, reagents were purchased from Sigma Aldrich, UK. All culture medium and fetal bovine serum (FBS) for cell or placental explant culture were obtained from Gibco (Gibco, Life Technologies, UK).

2.1 Placental collection

Wax blocks of archived villous explants from late first trimester placentas (8-12 weeks' gestation) were from previous work collected in the Saint Mary's Hospital, Manchester following maternal informed consent with approval from the Local Research Ethics Committee (Ref: 13/NW/0205; 08/H1010/55(+5)) (Farrokhnia et al., 2014).

For experiments using human term placentas, a favourable ethical opinion was given (Ref: 08/H1010/55+5) and written informed consent was obtained from all participants. All term placental samples were collected from Saint Mary's Hospital within 30 minutes of delivery of the placenta. Regarding infant birthweight, appropriate for gestational age (AGA) (n=14) was defined as the individualised birth weight ratios (IBR) between the 10^{th} to 90^{th} centile and FGR (n=14) was defined as an IBR below the 5^{th} centile (Gordijn et al., 2016). All participants were non-smokers. The exclusion criteria in our study included women with pregnancy complications, such as preeclampsia, chronic hypertension, diabetes, gestational diabetes, renal disease, collagen vascular disease, premature rupture of membranes, and pregnancies complicated with fetal or chromosomal abnormalities.

2.2 Placental explant model

2.2.1 Villous explants preparation

Human placental explants from healthy term pregnancies were prepared as previously described (Heazell et al., 2008). The procedure was aseptically performed in the tissue culture hood. After randomly selecting 6 different sites in the placenta, excluding sites of necrosis, infarction, and excessive tears, 6 chunks of villous tissue ($2cm^3$) were excised and placed into sterile, warm PBS solution. Placental tissue was further

washed in warm PBS twice to remove excess blood and dissected to obtain fragments of approximately $2mm^3$ / 5mg. Dry, sterile netwells (15mm diameter, 74µm mesh, Corning Inc, NY, USA) were inserted into the 12-well plates. Three villous tissue fragments, obtained from 3 different placental sites were pooled and added to netwells containing 1.5ml CMRL-1066 medium supplemented with 10% FBS, penicillin (100IU/ml), streptomycin (100µg/ml), L-glutamine (100mg/l), insulin (0.1mg/l), hydrocortisone (0.1mg/l), and retinol acetate (0.1mg/l) or DMEM/F12 culture medium supplemented with 10% FBS, 100IU/ml penicillin, 100µg/ml streptomycin sulfate and 2mM L-glutamine.

2.2.2 Hypoxic culture for villous explants

Explants were cultured in CMRL-1066 culture medium in 21% O_2 (5% CO_2, 95% air at 37°C) for 24 hours. Then, explants were divided into different treatment groups: atmospheric oxygen control (21% O_2), physiological oxygen control (6% O_2), hypoxia (1% O_2), or treated with cobalt chloride ($CoCl_2$; 200 micromoles (µM)) (Figure 2.1A) and cultured for up to 96 hours. $CoCl_2$ is a chemical that can mimic hypoxic conditions by activating the expression of hypoxia-inducible factor-1alpha (HIF-1alpha) (Manuelpillai et al., 2003, Kim et al., 2006). Media was collected throughout the culture period from day 2 to day 4 and stored at -20°C for assay of human chorionic gonadotrophin (hCG) and lactate dehydrogenase (LDH). The explants were denatured in 0.3M NaOH for assessment of protein content, or put into RNA later overnight for RNA isolation, or were stored in a clean eppendorf tube at -80°C for protein extraction or fixed in the neutral phosphate-buffered 4% formaldehyde solution overnight for immunostaining (Figure 2.1A).

2.2.3 Treatment with GSK-5182, DY131 or Bisphenol A

GSK-5182 is an analog of 4-hydroxytamoxifen (4-OHT) and is an inverse agonist, inhibiting the transactivation of ESRRG due to additional noncovalent interactions with Tyr-326 and Asn-346 of ESRRG's ligand binding domain (Chao et al., 2006). DY131

is a specific agonist of ESRRG, which can activate the transcription of ESRRG (Yu and Forman, 2005).

The concentration of GSK-5182 or DY131 applied to the term placental explants ranged from 5μM to 50μM, and the concentration of BPA ranged from 1pM to 1μM. Villous explants were set up at day 0 and GSK-5182, DY131, or BPA were supplemented in the DMEM/F12 culture medium immediately and were cultured in the incubator with 21% O_2 (5% CO_2, 95% air at 37°C) for up to 48 hours. Conditioned culture medium and placental explants were harvested after 24 hours or 48 hours of culture.

For the explants cultured for 96 hours, all explants were cultured in 21% O_2 for 24 hours. Then half of the explants were moved into hypoxic conditions (1% O_2) and the remaining explants were cultured in 21% O_2 for 24 hours. DY131 or GSK5182 were then added into the medium and explants were cultured in 21% O_2 or 1% O_2 for another 48 hours until harvest (Figure 2.1B).

2.2.4 Transfection of placental explants with siRNA or miRNA mimics using DharmaFECT transfection reagent

Small interfering RNAs (siRNAs) and miRNAs are produced as double-stranded RNA precursors, which are cleaved by the double-stranded specific endonuclease, Dicer, which can further guide the RNA-induced silencing complex (RISC) to specifically cleave the sequence of interest (Fire et al., 1998, Hammond et al., 2000, Bartel, 2004, Baek et al., 2008). The transfection protocol for the miR-377 precursor mimetic (Ambion, USA), non-targeting miRNA control (Ambion, USA), the siRNAs targeting ESRRG (Dharmacon, UK), and non-targeting siRNA control (Dharmacon, UK) was developed according to our previous study, and performed using the lipid-based carrier, DharmaFECT 2 (Dharmacon, GE Healthcare, UK), to introduce siRNAs or specific miRNA precursor mimetics into cells or villous explants (Forbes et al., 2009).

A 200µl transfection mixture was prepared containing DharmaFECT 2 and specific siRNAs (or siRNA controls, or the miR-377 precursor mimetic, or miRNA controls), and was incubated in the culture hood for 20 minutes at room temperature. 1.3ml culture media (1:1 DMEM/F12) without FBS or antibiotics, was mixed with the transfection mixture and transferred to 12-well plates containing the villous explants. The explants were maintained at 37°C in 21% O_2 in a humidified incubator for 8 hours. After removing the culture media and rinsing the villous explants with warm PBS + Ca/Mg twice, 1.5ml fresh culture media containing 10% FBS and antibiotics was added. The culture media was changed every 24 hours, and the explants were harvested after a total of 48 hours' culture. No specific effects of the negative control siRNAs on first trimester villous explants have previously been observed (Farrokhnia et al., 2014). The explants were either transferred into RNA later or fixed in neutral phosphate-buffered formaldehyde solution (4% NBF v/v), were incubated overnight and were processed for RNA collection, or immunohistochemistry, respectively.

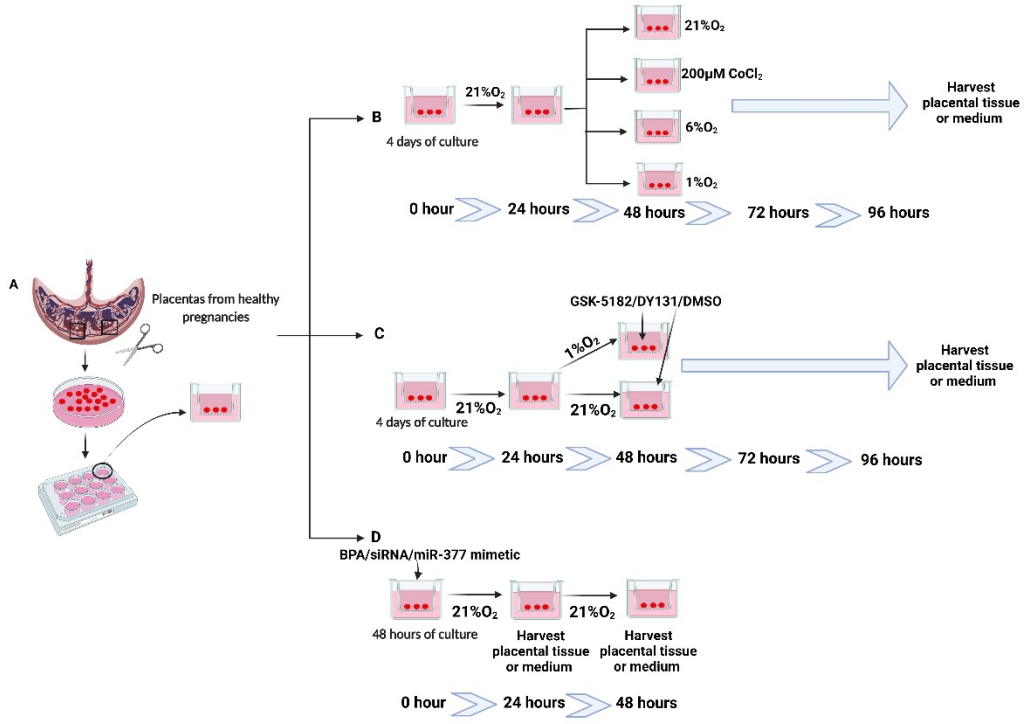

Figure 2.1 A schematic of the villous explant culture experiments.

(A) Villous explants were prepared from the term placentas of healthy pregnancies. (B) Villous explants were cultured under different oxygen levels (1% O_2, 6% O_2, or 21% O_2) or treated with 200μM $CoCl_2$. (C) Placental explants were treated with GSK-5182 or DY131 (20μM or 50μM), or 0.075% dimethyl sulfoxide (DMSO), and were cultured at 21% O_2 or 1% O_2 for 96 hours. (D) Term placental explants were set up at day 0 and treated with BPA (from 1pM to 1μM), 0.05% (v/v) ethanol (control group), siRNAs, siRNA controls, a miR-377 mimetic, or miRNA controls for 24 hours or 48 hours of culture. Image created with BioRender.com.

2.2.5 Incubation of placental explants with siRNA or miRNA mimics without transfection reagent

According to previous work in our laboratory, incubation of siRNAs in the medium of cultured explants is effective to transport the siRNAs into the syncytiotrophoblast in the absence of transfection reagents (Forbes et al., 2009). siRNAs, a non-targeting siRNA control, miR-377 mimics, or a non-targeting miRNA control were directly added to culture media in 12-well plates, in a total volume of 2ml. After gently mixing the culture media, 3 placental explants were cultured in each netwell at 37°C in a humidified incubator at 21% O_2 (with 5% CO_2/ balanced N_2) for 48 hours; the culture media fully covered the villous explants. After 48 hours, villous explants were collected for RNA extraction and immunohistochemistry.

2.3 BeWo cell culture

2.3.1 Preparation for cell culture

DMEM/F12 culture medium supplemented with 10% FBS, 100IU/ml penicillin, 100µg/ml streptomycin sulfate and 2mM L-glutamine was used for cell culture. After removal from storage in liquid nitrogen, BeWo cells (choriocarcinoma cells; ATCC, CCL-98) were passaged a maximum of 25 times and cultured in a 37°C incubator at 21% O_2 with a humidified atmosphere of 5% CO_2 in air. The starting passage for BeWo cells is 18 times. When BeWo cells were 90% confluent, the cells were detached from the flask using 0.05% Trypsin-EDTA. The addition of 5ml warm culture media containing 10% FBS neutralized the effect of the trypsin-EDTA; the cell suspension was transferred into a 50ml sterile tube, centrifuged for 5 minutes at 100*g* at room temperature and the cell pellet was resuspended with 10ml of warm culture media. Cells were counted using a haemocytometer and cells were plated at a density of 1.0 x 10^7 cells/ml in flasks or at 1.0 x 10^6 cells/well in 12-well plates containing 1.5ml media. Culture media was renewed every 24 hours.

2.3.2 Transfection of BeWo cells

The lipid-based transfection protocol was performed according to a previous study (Forbes et al., 2009). The transfection details were described in section 2.2.4. Briefly, BeWo cells were seeded into 12-well plates (1.0 x 10^6 cells per well) and cultured at 37°C in a humidified chamber under a 5% CO_2/95% air gas mixture overnight. BeWo cells were cultured to 70% confluency, transferred to DMEM/F12 culture medium without FBS or antibiotics and the transfection mix was added. After 8 hours of culture, the media was removed, cells were washed in PBS-Ca/Mg and 1.5ml of fresh culture media containing fetal calf serum and antibiotics was added.

The effect of transfection with miR-377 pre-miR precursors or *ESRRG* siRNAs was compared with the control groups, including untreated cells (untreated control), BeWo cells exposed with DharmaFECT 2 transfection reagent alone (mock), or cells transfected with non-targeting siRNAs or a non-targeting miRNA control. These controls provided a baseline for evaluating the effects of *ESRRG* siRNAs or miRNA mimics on mRNA or protein expression and cell function. The *ESRRG* siRNA concentrations tested were 20nM, 50nM, 100nM, and 200nM and the mRNA expression of *ESRRG* was measured after 24, 48 or 72 hours of culture.

2.4 RNA extraction

2.4.1 Sample preparation

2.4.1.1 BeWo cells

BeWo cells were rinsed twice with cold PBS. QIAzol lysis buffer (700 µl per well; miRNeasy mini kit, QIAGEN, Germany) was added to cells, which were detached from the plate using a cell scraper. Cell suspensions were transferred into eppendorf tubes and stored at -80 °C immediately, until RNA extraction was performed.

2.4.1.2 Villous explants

Fresh placental tissue or cultured explants were placed into eppendorf tubes which contained 1ml RNA later (Sigma, UK) and were incubated at 4 °C overnight. The tissues

were then weighed and approximately 50 µg was transferred into new eppendorf tubes for homogenization. The homogenizer (SHM1, UK) and eppendorf tubes (1.5ml and 0.6ml) were autoclaved before use. A Qiagen RNA extraction kit was used to extract total RNAs including small RNAs (miRNeasy mini kit, QIAGEN, Germany) from the homogenates, following the manufacturer's instructions. Briefly, 700µl QIAzol lysis buffer was added to the samples for villous tissue disruption and homogenization was immediately performed on full power for 30 seconds on ice.

2.4.2 DNase digestion and RNA collection

After the tissue was uniformly homogenized or BeWo cells lysates were thawed, chloroform (VWR International, USA) was added into the tubes with vigorously shaking for 15 seconds. The tubes were then centrifuged at 12,000*g* at 4°C for 15 minutes. The upper aqueous phase was transferred into a new collection tube, which contained a 1.5 volume of 100% ethanol. A DNase kit (QIAGEN, Germany) was used for the DNase digestion procedure. RPE buffer was used to wash the column membrane, then 30µl RNase-free water was pipetted directly into the column membrane, which was centrifuged at 8000*g* for 1 minute. When the pure RNA was collected, all samples were stored on ice and a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) was used to assess the concentration and quality of the RNA. A ratio measurement of A260/280 close to 2 was considered sufficient RNA purity for downstream applications.

2.4.3 Reverse transcription-polymerase chain reaction (RT-PCR)

mRNA: A total of 500ng-2500ng RNA (depending on the RNA concentration extracted) from each sample was converted to cDNA using an AffinityScript Multiple Temperature cDNA Synthesis kit (Agilent, USA) and all procedures followed the first-strand cDNA synthesis protocol. The PCR primers for *ESRRG*, its downstream genes and *RPLP0* (60S acidic ribosomal protein P0, Eurofins, UK) are listed in Table 2.1. *RPLP0* was used as a house-keeping gene and an endogenous control for mRNA expression, as *RPLP0* has previously been shown to be stably expressed in human term placentas

(Luo et al., 2014). The powerup SYBR Green Master Mix (Thermo Fisher Scientific, USA) was used for the PCR reaction and the Applied Biosystems Step-one system (Thermo Fisher Scientific, USA) was used to run the reaction. The procedure was 50°C for 2 minutes (UDG activation), 95°C for 2 minutes (Dual-lock DNA polymerase), and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The conditions for the melt curve analysis included 95°C for 15 seconds, 60 °C for 1 minute and 95°C for 15 seconds.

The representative standard curve, amplification plots, and melt curve are shown in Figure 2.2. The standard curve was used to test the efficiency of the primers used in this study (Figure 2.2A). The correlation coefficient (R^2) reflects the linearity of the standard curve and is a measure of how well the data fit the standard curve; ideally, this value should be close to 1 (Figure 2.2A). As shown in the Figure 2.2A, five separate cDNA dilution series (1:2 dilution) were prepared. An ideal PCR reaction for the primers should have an efficiency between 90% and 110%. An amplification plot showed the accumulation of product over the duration of the entire PCR reaction (Figure 2.2B). The melt curve shows the dissociation characteristics of double-stranded DNA during heating, and a single peak in the melt curve indicates the presence of a single amplicon; this was verified by the presence of a single PCR product (Figure 2.2C).

miRNA: A miRCURY LNA RT kit was used for reverse transcription of miRNAs (10ng) according to the quick-start protocol. The primer sequences for the miRNAs and endogenous reference genes, U6 (RNU6‑1) snRNA (Dharmacon, lnc., USA) are listed in Table 2.1. The miRCURY LNA SYBR Green PCR kit (QIAGEN, Germany) was used to measure the expression of the miRNAs. The PCR procedure included an initial heat activation (95 °C for 2 minutes), and 40 cycles of 95°C for 10 seconds (denaturation), and 56°C for 60 seconds (combined extension). The melt curve was performed by following the manufacturer's protocol (miRCURY LNA SYBR Green PCR kit): 95°C for 15 seconds, 60 °C for 1 minute, and 95°C for 15 seconds.

Comparative quantification was undertaken using the $2^{(-\triangle\triangle Ct)}$ method for the genes and miRNAs of interest. The relative expression of mRNA or miRNAs was normalised to the endogenous control and determined using the $2^{(-\triangle\triangle Ct)}$ method where $\triangle Ct$ =Ct (target genes) – Ct (housekeeping gene) and $\triangle\triangle Ct = \triangle Ct$ (treatment group) $-\triangle Ct$ (control group)

Table 2.1 mRNA and miRNA primer sequences

Primer set	Gene name	Accession number	Annealing temperature (°C)	Primer Sequence (5'-3') Forward (F), Reverse (R)
mRNA	Gene name			
ESRRG	Oestrogen related receptor gamma	NM_001438	60	F: 5'-CTG ACG GAC AGC GTC AAC C-3' R: 5'-GGC GAG TCA AGT CCG TTC TG-3'
RPLP0	Ribosomal Protein Lateral Stalk Subunit P0	NM_001002	60	F: 5'-TGC ATC AGT ACC CCA TTC TAT CA-3' R: 5'-AAG GTG TAA TCC GTC TCC ACA GA-3'
HSD17B1	Hydroxysteroid 17-Beta Dehydrogenase 1	NM_000413	60	F: 5'-GCC TTC ATG GAG AAG GTG TT-3' R: 5'-CGA AAG ACT TGC TTG CTG TG-3'
CYP191.1	Cytochrome P450 Family 19 Subfamily A Member 1	NM_000103	60	F: 5'-ACG GAA GGT CCT GTG CTC G-3' R: 5'-GTA TCG GGT TCA GCA TTT CCA-3'

PLAC1	Placenta-specific protein 1	NM_021796	60	F: 5’-ATT GGC TGC AGG GAT GAA AG-3’ R: 5’-TGC ACT GTG ACC ATG AAC CA-3’
HSD11B2	Hydroxysteroid 11-Beta Dehydrogenase 2	NM_000196	60	F: 5’-GAC CTG ACC AAA CCA GGA GA-3’ R: 5’-GCC AAA GAA ATT CAC CTC CA-3
miRNAs		miRbase Accession Number		Target sequence
U6			56	GGGCAGGAAGAGGGCCTAT
hsa-miR-377		MIMAT0000730	56	AUCACACAAAGGCAACUUUUGU
hsa-miR-204-5p		MIMAT0000265	56	UUCCCUUUGUCAUCCUAUGCCU
hsa-miR-30a-5p		MIMAT0000087	56	UGUAAACAUCCUCGACUGGAAG
hsa-miR-26a-5p		MIMAT0000082	56	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-23-3p		MIMAT0000078	56	AUCACAUUGCCAGGGAUUUCC
hsa-miR-34b-5p		MIMAT0000685	56	UAGGCAGUGUCAUUAGCUGAUUG

hsa-miR-30a	MIMAT0000087	56	UGUAAACAUCCUCGACUGGAAG

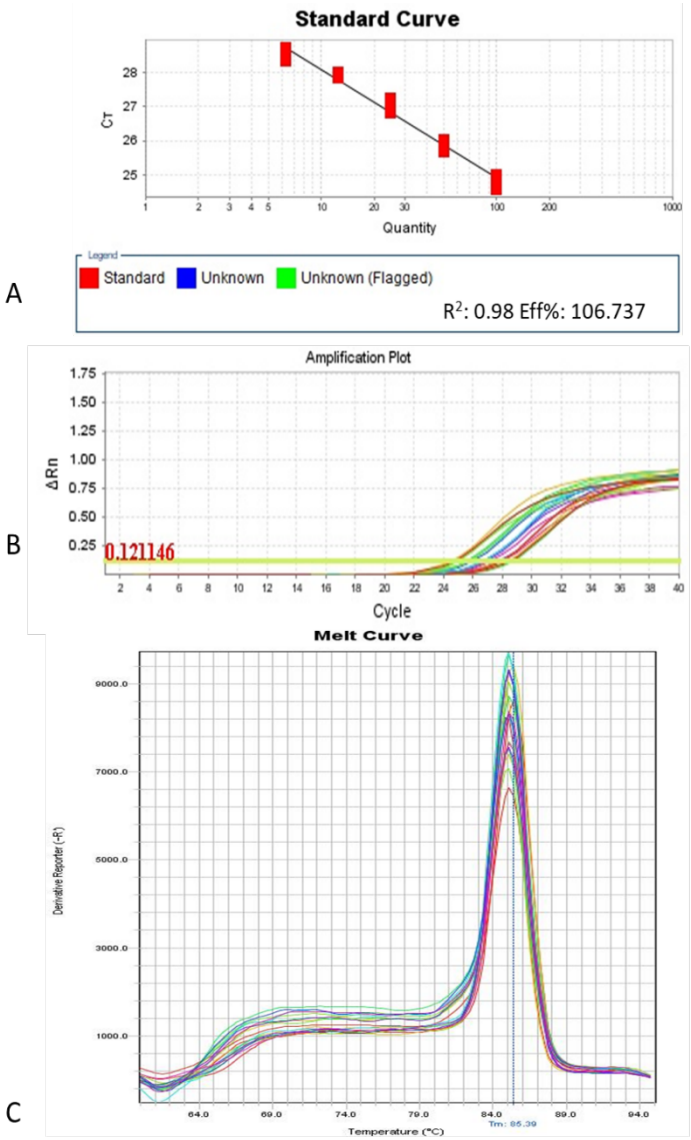

Figure 2.2 Representative mRNA RT-PCR experiment to measure *HSD17B1*.

(A) A standard curve for the hydroxysteroid 17-beta dehydrogenase 1 (*HSD17B1*) primer; y-axis is the threshold cycle (Ct), and the x-axis shows the quantity of the cDNA target. (B) Amplification plots showing the normalised fluorescence for individual wells during the thermal cycling protocol. (C) Melt curve showing the temperature at which 50% of DNA is denatured.

2.5 Protein extraction

2.5.1 Sample Preparation

BeWo cells: BeWo cells were washed twice in PBS and scraped into RIPA lysis buffer (Sigma, UK) supplemented with a protease inhibitor complex (P8340, Sigma, 10µl/ml), phosphatase inhibitor II (P5726, Sigma, 10µl/ml), and phosphatase inhibitor III (P0044, Sigma, 10µl/ml).

Placental tissue: 100 µg of tissue was homogenized in 500µl RIPA lysis buffer containing the protease and phosphatase inhibitor complex, using a homogeniser (SHM1, UK) at full power for 30 seconds. Lysates were then incubated on ice for 15 minutes and were centrifuged at 4^{o}C and 11,600*g* for 5 minutes. The supernatant was then transferred to a fresh eppendorf tube.

2.5.2 Bicinchoninic Acid (BCA) protein assay

A BCA protein assay kit (Product No.23225, Thermo Fisher Scientific, USA) was used to quantify the protein concentration of the fresh placental tissue, explants or BeWo cell lysates. All protein samples were diluted 1:20 in water and put on ice. Serial dilution of bovine serum albumin (BSA) was performed to generate protein standards. The working reagent was prepared according to the instructions: 50 parts of reagent A and 1 part of reagent B were mixed (Reagent A: Reagent B=50:1). After pipetting 25 µl of samples or BSA standards into a 96 well plate, 200µl of working reagent was added to each well, and plates were placed on a shaker for 30 seconds. The plates were then covered and incubated at 37°C for 30 minutes. The absorbance was measured in the plate reader (Omega Plate Reader, BMG LABTECH, UK) at 562nm, and all samples or protein standards were run in triplicate wells. The standard curve was plotted using GraphPad Prism v.8.0.1 and total protein content was calculated by interpolating sample absorbances from the standard curve.

2.6 Western blotting

A Sodium-Dodecyl Sulphate polyacrylamide (SDS-PAGE) gel was prepared that contained a 10% resolving gel and a 3% stacking gel, based on the protein molecular weight of ESRRG (51kD). 30µg of fresh placental tissue or 120µg of villous explants supernatants or 30µg cell protein supernatants were loaded into each well of the 3% stacking gel. The gel was run at 120V for 80 minutes, then was cut and transferred to a PVDF membrane (Millipore, Sigma, UK) at 120V for 80 minutes. The whole PVDF membrane was rinsed in 3% (w/v) milk (Marvel, UK) in 1x PBS for 1 hour at room temperature, then incubated with a monoclonal ESRRG (ab128930, 0.12µg/ml Abcam, UK) primary antibody diluted in 0.1% PBST (PBS-Tween-20) at 4°C overnight. beta-tubulin (ab6046, 0.1µg/ml Abcam, UK) or beta-actin (20536-1-AP, 0.09µg/ml, Proteintech, UK) was used as the house-keeping gene since its expression was not different between treatment groups.

After washing with PBS for 5 minutes once, 0.1% PBST for 5 minutes twice, and PBS for 5 minutes once, the PVDF membrane was incubated with a fluorescently conjugated secondary antibody (IRDye green, 0.5µg/ml, LI-COR Bioscience, USA) at room temperature for 1 hour. After another round of washing with PBS and 0.1% PBST, the PVDF membrane was transferred to a falcon tube containing PBS; the resulting blot was scanned using a Licor Odyssey (LI-COR Bioscience, USA) and Image studio lite software (Image studio lite version 5.2, LI-COR Bioscience, USA) was used to analyse the bands. The target protein expression was normalised to expression of the housekeeping protein.

2.7 Immunostaining

2.7.1 Fixation and processing of placental tissue

Placental explants were collected at day 4 and fixed in 4% neutral buffered formalin overnight at 4°C, followed by rinsing 3 times by PBS. Archived first-trimester placental wax blocks were also analysed for comparison; 5µm sections were cut by microtome

(Leica RM2245, Germany) and transferred onto microscope slides pre-coated with poly-L-lysine.

2.7.2 Immunohistochemical staining of placental tissue

The paraffin sections were heated at 60°C for 10 minutes and dewaxed in Histoclear (Fisher Scientific, UK) 3 times for 5 minutes each, then were passed through a series of ethanol solutions from 100% (v/v) to 70% (v/v). Slides were placed in running tap water for 5 minutes, then were treated for antigen retrieval by microwave boiling for 5 minutes twice at full power (800w) in 0.01M citrate buffer (pH 6.0). After incubation in the hot buffer for 20 minutes, slides were immersed in tap water, then incubated with 3% (v/v) hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Slides were incubated with the non-immune block (10% (v/v) goat serum and 2% (v/v) human serum in 0.1% (v/v) TBST (TBS-Tween-20)) for 30 minutes at room temperature, to prevent non-specific binding of antibodies. Without washing, sections were incubated with a monoclonal antibody against HIF-1alpha, polyclonal antibody against ESRRG, a monoclonal antibody against Ki-67, or a monoclonal antibody against M30, overnight at 4°C. Ki-67 is a nuclear antigen and is a proliferative marker, which is expressed in all active phases of the cell cycle, not expressed in the stationary G0 phase (Li et al., 2015a). M30 is a marker for the detection of apoptotic cells as anti-M30 monoclonal antibody reacts with a caspase-cleaved product of keratin 18 (Leers et al., 1999). The concentration of the primary antibodies is listed in Table 2.2; antibodies were diluted in the non-immune block. The negative control was an isotype-specific non-immune rabbit or mouse IgG from Sigma, diluted in the non-immune block to the same concentration as the primary antibodies.

After washing the sections once with TBS, twice in 0.6% TBST, and once in TBS for 5 minutes, the secondary antibody was applied, and the slides were incubated for 30 minutes at room temperature. The sections were washed with TBS in the same sequence as above; avidin-peroxidase (5µg/ml) was then added to the slides for 30

minutes, to bind the biotinylated secondary antibody. Chromogenic substrate diaminobenzidine (DAB; Sigma-Aldrich, UK) was applied to the sections for between 2-10 minutes; colour development was monitored under the microscope. After washing with dH_2O and incubating for 5 minutes, all slides were counterstained with Harris's hematoxylin (Sigma-Aldrich, UK) for 5 minutes, rinsed in tap water, then treated with acid alcohol for 3 seconds, and rinsed in hot tap water. The sections were dehydrated in increasing concentrations of ethanol (70%, 95%, and 100% (v/v)) and Histoclear 3 times before mounting in DPX (Sigma-Aldrich, UK).

2.7.3 Image analysis

For villous explants, 10 random fields of view per each section were captured by an Olympus Microscope (Olympus, UK). All images were analysed using QuPath (version 0.2.3, developed by University of Edinburgh (Bankhead et al., 2017)).

2.7.3.1 Analysis of the number of cells in the cycle and cell apoptosis in villous explants

Ki-67 was used as a marker of cell proliferation and M30 was used as an apoptotic marker; both have been widely used in analysis of placental samples and cultured villous explants (Heazell et al., 2008, Matos et al., 2014, Renshall et al., 2021). Quantification of the positive nuclei or areas of Ki-67 and M30 staining was achieved by counting total haematoxylin-stained nuclei, and the number of cells / areas positive for DAB staining. The total nuclei were quantified using QuPath, and the number Ki-67 (positive nuclei) (Figure 2.3 A-B) or M30 positive cells (cytoplasmic staining) were counted manually (Figure 2.3 C-D). The number of nuclei positively stained by the primary antibody were presented as a proportion of the total nuclei, and the mean proportion was calculated to present as the proliferation index or apoptotic index.

2.7.3.2 Analysis of ESRRG protein staining in villous explants

Quantification of ESRRG was achieved by quantifying the DAB positive area and the total tissue area using QuPath. The threshold for detecting DAB area and total tissue area was created in QuPath, and the DAB channel or haematoxylin channel was selected for DAB area detection or tissue area detection separately. The proportion of ESRRG staining area in the villous explants indicates the proportion of ESRRG staining (Figure 2.3 E-F).

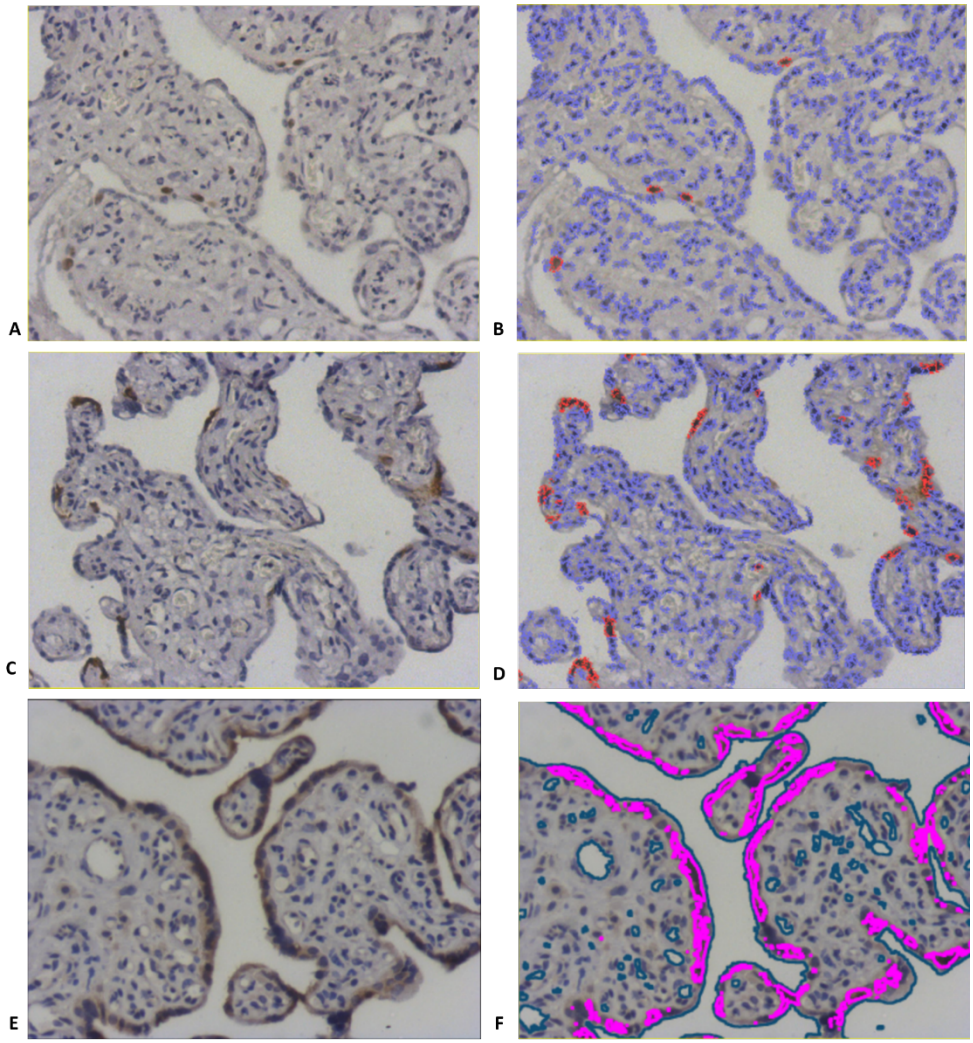

Figure 2.3 Representative images of quantification by QuPath software.

(A-B) Ki-67 staining. (C-D) M30 staining. In images B and D, the overlaid blue colour indicates the cell nuclei and the overlaid red colour indicates cells/area with Ki-67 or M30 positive staining. (E-F) representative images of ESRRG staining in the villous explants. (F) the overlaid pink colour indicates the DAB-positive area, and the overlaid blue colour indicates the total tissue area.

Table 2.2 Primary and secondary antibodies used for immunohistochemistry

Primary antibody	Concentration of primary antibody	Secondary antibody	Concentration of secondary antibody
HIF-1alpha (Abcam 51608, rabbit monoclonal anti-HIF-1alpha, Abcam, UK)	10µg/ml	Polyclonal Goat anti-rabbit Biotinylated (Dako, UK)	3.85 µg/ml
ESRRG (Abcam 215947, rabbit polyclonal anti-ESRRG, Abcam, UK)	10µg/ml	Polyclonal Goat anti-rabbit Biotinylated (Dako, UK)	3.85 µg/ml
Ki-67 (monoclonal antibody against Ki-67, Dako, UK)	0.174µg/ml	Polyclonal Goat anti-mouse Biotinylated (Dako, UK)	3.85 µg/ml
M30 (monoclonal antibody against M30, Roche, UK)	0.132µg/ml	Polyclonal Goat anti-mouse Biotinylated (Dako, UK)	3.85 µg/ml

2.8 ELISA for quantification of hCG secretion

hCG was detected in explant-conditioned culture medium using an ELISA kit (DRG Diagnostics, Marburg, Germany) according to the manufacturer's instructions. A standard curve was made using recombinant hCG provided in the kit. Briefly, 25µl of each standard, control, and sample were dispensed into each well in duplicate. 100µl enzyme conjugate was added into each well with mixing for 10 seconds, then incubation at room temperature for 30 minutes. Afterward, the contents of the wells were briskly shaken out; wells were rinsed 5 times using distilled water. After removing the residual droplets in the wells, 100µl substrate solution was added into each well, followed by incubation for 10 minutes at room temperature. Then, 50µl stop solution was added to stop the enzymatic reaction and the absorbance of each well at 450+/-10nm was determined using a plate reader (Omega Plate Reader, BMG LABTECH, UK).

2.9 Quantification of LDH release

The level of LDH in the explant-conditioned culture medium was measured using a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany), following the instruction of the manufacturer. 100µl culture medium was pipetted into 96 wells in duplicate and 100µl of freshly prepared reaction mixture was added into each well, followed by incubation for 30 minutes at room temperature. 50µl stop solution was applied into the wells and the plate was shaken for 10 seconds. The plate reader (Omega Plate Reader, BMG LABTECH, UK) was used to measure the absorbance of samples at 490-492nm. LDH release was considered a proxy measure of necrosis in the explants.

2.10 BioRad protein assay

Villous placental explants were lysed in 0.3M NaOH and a protein assay was used to quantify protein content. Three explants were added into 4ml of 0.3M NaOH and incubated 37°C overnight. Before the protein assay, the BioRad dye reagent (Bio-Rad Protein Assay, BioRad, USA) was removed from the fridge to warm to the room temperature. A 0.25mg/ml stock protein solution was prepared with BSA diluted in 0.3M NaOH, which was used to make serial dilutions for the standard curve. 20µl standard or sample were added in a 96 wells plate, and then 180µl neutralising solution (1:1.25 of 0.3M NaOH and 0.3M HCl) were applied. 50µl BioRad dye reagent were added to each well. The plate was read on the plate reader (Omega Plate Reader, BMG LABTECH, UK) at 595nm to measure the absorbance of samples.

2.11 The measurement of BPA concentration in human placental tissue

The ELISA kit for measuring BPA levels was purchased from Abcam (ab175820, Abcam, UK).

2.11.1 Sample preparation

Sample preparation was undertaken according to the protocol from Abcam (ab175820, Abcam, UK). Briefly, fresh placental tissues were stored at -80°C immediately prior to use. Placental tissue (approximately 1gram) was homogenised in 4ml water using a homogeniser (SHM1, UK) at room temperature, then 8µl acetic acid was added to acidify the homogenate. After adding 4ml ethyl acetate, the homogenate was vortexed thoroughly and centrifuged at 12,000*g* for 3 minutes. The organic phase was collected in a new tube, and this extraction procedure was repeated twice. The organic phase was dried with nitrogen gas in the hood, and the dried residue was dissolved in 20µl ethanol (96%, v/v), with the addition of 500µl sample dilution buffer. The tubes were centrifuged at 10,000*g* for 5 minutes and then the supernatant was moved into a new tube.

2.11.2 ELISA for BPA measurement

All the reagents, including the wash buffer, HRP-conjugate, sample dilution buffer, and BPA standards were prepared following the manufacturer procedure. 100µl of sample or standard were transferred to a 96 well plate in duplicate and 100µl HRP-conjugate solution was added to the wells; the plate was then incubated for 2 hours at room temperature. After washing the wells three times with washing buffer, 200µl TMB substrate was added to the wells and the plate was incubated for 30 minutes. 50 µl 2 N sulfuric acid was added into the wells and the plate was read at 450nm immediately. The wells with 200µl sample dilution buffer were considered as the blank control and the wells with 100µl sample dilution buffer with 100µl HRP-conjugate were considered as the maximum binding control wells; values from these wells were used for normalising the absorbance values of the standards and samples. The standard curve was made according to the protocol and the BPA concentration was calculated based on the standard curve.

2.12 Statistical analysis

The Shapiro-Wilk test was used to assess whether data were normally distributed. When the data were normally distributed in two unmatched groups, statistical significance was tested using an unpaired t test; otherwise, a Mann-Whitney Test was used. Normally distributed data with three or more paired groups was analysed by one-way ANOVA; a Friedman multiple comparison test was used to analyse paired, non-parametric data. Unpaired, non-parametric data with three or more groups was analysed using a Kruskal-Wallis test. $P<0.05$ was considered as indicative of statistical significance. All data are presented as the mean ± standard deviation (SD) (normally distributed) or median+/-interquartile range (IQR) (non-normally distributed) depending on data distributions with n=number of placentas; GraphPad Prism version 8.0.1 (GraphPad Software, USA) was used to undertake the statistical analysis.

3 CHAPTER 3: Placental expression of Oestrogen Related Receptor gamma (*ESRRG*) is reduced in FGR pregnancies and is mediated by hypoxia

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Abstract

Fetal growth restriction (FGR) describes a fetus which has not achieved its genetic growth potential, it is closely linked to placental dysfunction and uteroplacental hypoxia. Oestrogen related receptor gamma (*ESRRG*) is regulated by hypoxia and is highly expressed in the placenta. We hypothesised *ESRRG* is a regulator of hypoxia-mediated placental dysfunction in FGR pregnancies. Placentas were collected from women delivering appropriate for gestational age (AGA; n=14) or FGR (n=14) infants. Placental explants (n=15) from uncomplicated pregnancies were cultured for up to 4 days in 21% or 1% O_2, or with 200μM cobalt chloride ($CoCl_2$), or treated with the *ESRRG* agonists DY131 under different oxygen concentrations. RT-PCR, Western blotting and immunochemistry were used to assess mRNA and protein levels of ESRRG and its localisation in placental tissue from FGR or AGA pregnancies, and in cultured placental explants. ESRRG mRNA and protein expression were significantly reduced in FGR placentas, as was mRNA expression of the downstream targets of *ESRRG*, *HSD11B2*, and *CYP19A1.1*. HIF-1alpha protein localised to the nuclei of the cytotrophoblasts and stromal cells in the explants exposed to $CoCl_2$ or 1% O_2. Both hypoxia and $CoCl_2$ treatment decreased *ESRRG* and its downstream genes' mRNA expression, but not ESRRG protein expression. DY131 increased expression of ESRRG signalling pathways and prevented abnormal cell turnover induced by hypoxia. These data demonstrate that placental *ESRRG* is hypoxia-sensitive and altered *ESRRG*-mediated signaling may contribute to hypoxia-induced placental dysfunction in FGR. Furthermore, DY131 could be used as a novel therapeutic approach for the treatment of placental dysfunction.

3.1 Introduction

Fetal growth restriction (FGR) describes a fetus which does not reach its genetic growth potential and affects between 5 and 10% of pregnancies. FGR is important as it has both short and long-term consequences including: stillbirth, neonatal death, neurodevelopmental delay in childhood, or increased cardiovascular or metabolic disorders in adulthood (Williams et al., 1982, Barker, 1992, Pels et al., 2019). The majority of cases of FGR are mediated by abnormal placental structure and function (Macara et al., 1996). In normal placentas, terminal villi are covered by syncytiotrophoblast and subjacent cytotrophoblast; the proliferation and fusion of cytotrophoblast continuously renews the overlying syncytiotrophoblast to support normal placental function (Jones and Fox, 1991). Reduced remodeling of maternal uterine spiral arteries into low resistance vessels in early pregnancy is hypothesized to cause poor oxygen (O_2) delivery to the intervillous space, and lead to placental hypoxia. This is thought to contribute to the pathogenesis of FGR, particularly in early-onset and severe cases which are characterised by aberrant villous trophoblast turnover and placental dysfunction (Macara et al., 1996, Genbacev et al., 1996, Levy et al., 2002). Indeed, we have previously demonstrated that rates of apoptosis are increased and proliferation is decreased in placental explants cultured in hypoxic conditions (1% O_2), which is reminiscent of the disordered cell turnover observed in FGR placentas (Heazell et al., 2008, Heazell et al., 2011).

Oestrogen-related receptor-gamma (*ESRRG*) is a gene that encodes the orphan nuclear receptor, ESRRG. It is highly expressed in metabolically active organs, including kidney, heart, liver and human placenta (Takeda et al., 2009, Poidatz et al., 2012). In the placenta, the ESRRG protein is mainly located in the villous trophoblast, with the highest expression in syncytiotrophoblast (Kumar and Mendelson, 2011, Poidatz et al., 2012). Luo et al. (Luo et al., 2014) reported that both mRNA and protein expression of ESRRG are increased in human placentas from pregnancies complicated by preeclampsia and may be involved in regulating blood pressure homeostasis in mice.

Conversely, both mRNA and protein expression of ESRRG were decreased in placentas from pregnancies complicated by FGR (Poidatz et al., 2015a, Zhu et al., 2018a). *ESRRG* is also reported to regulate trophoblast proliferation, differentiation, and invasion (Kumar and Mendelson, 2011, Zhu et al., 2018a). siRNA-mediated silencing of ESRRG expression inhibited proliferation and invasion of the trophoblast cell line, HTR-8/SVneo, (Zhu et al., 2018a), and decreased differentiation of primary cytotrophoblast into syncytiotrophoblast (Kumar and Mendelson, 2011). Based on this evidence, it is plausible that *ESRRG* may play a role in regulating trophoblast function, and dysregulation of *ESRRG* may contribute to the placental dysfunction observed in FGR. Presently, there is no effective treatment for FGR and a better understanding of the pathophysiological mechanism(s) which drive placental dysfunction in FGR placentas, is key for therapeutic advances to be made. Therefore, understanding the mechanisms regulating *ESRRG* in FGR may help to identify new therapeutic targets.

There is some evidence that *ESRRG* expression is regulated by hypoxia; when second trimester primary cytotrophoblasts were cultured at 2% O_2, both the mRNA and protein levels of ESRRG were decreased and the rate of differentiation into syncytiotrophoblast was reduced (Kumar and Mendelson, 2011). Furthermore, siRNA-mediated knockdown of hypoxia-inducible factor 1-alpha (HIF-1alpha encoded by the gene, *HIF-1A*) induced the expression of *ESRRG* and its downstream gene *CYP191.1* (cytochrome P-450) (Kumar and Mendelson, 2011). Other downstream genes of *ESRRG* in placental cells include hydroxysteroid 17-beta dehydrogenase 1 (*HSD17B1*), placenta-specific protein 1 (*PLAC1*), and hydroxysteroid 11-beta dehydrogenase 2 (*HSD11B2*) (Luo et al., 2013, Zhu et al., 2018a). These products are also altered in the placenta in response to hypoxia supporting a role for *ESRRG* signaling in the placental response to hypoxia (Heiniger et al., 2003, Homan et al., 2006, Devor et al., 2021).

The *ESRRG* pathway can also be manipulated pharmacologically. GSK5182, a 4-OHT analog, is a highly selective inverse agonist of *ESRRG*, due to its additional noncovalent interactions with Y326 and N346 at the active site of *ESRRG* (Chao et al., 2006, Vernier

et al., 2020). DY131 is an agonist for *ESRRG* which has been used in primary cytotrophoblast and animal models to activate the expression of *ESRRG* (Yu and Forman, 2005, Kumar and Mendelson, 2011, Luo et al., 2014, Poidatz et al., 2015b). Although past studies have shown reduced expression of *ESRRG* in FGR placentas (Poidatz et al., 2015a, Zhu et al., 2018a), there is little knowledge as to how dysregulated *ESRRG* signalling is linked to the placental dysfunction observed in FGR (Zou et al., 2021). Moreover, the potential for modulation of *ESRRG*-pathway has not been explored in the placental explant model. In this study, we hypothesised that the reduced expression of *ESRRG* observed in FGR is mediated by hypoxia, and that agonists of *ESRRG* could rescue hypoxic changes in cultured placental explants.

3.2 Materials and Methods

All the reagents were obtained from Sigma (Sigma, UK) unless specified.

3.2.1 Placental collection

This study was approved by the Local Research Ethics Committee (08/H1010/55+5) and written informed consent was obtained from all participants in Saint Mary's Hospital, Manchester. All births included in this study were by Caesarean section to eliminate the effects of oxidative stress which occur during normal labour.

In our study, appropriate for gestational age (AGA) (n=14) was defined as an individualised birth weight ratio (IBR) between the 10^{th} to 90^{th} centile and FGR (n=14) was defined as an IBR below the 5^{th} centile. Participant characteristics are shown in Table 3.1. Women with pregnancy complications such as preeclampsia, chronic hypertension, renal disease, diabetes, gestational diabetes, collagen vascular disease, premature rupture of membranes and pregnancies complicated with fetal anomalies or chromosomal abnormalities were excluded from this study. Term placentas (n=15) from uncomplicated pregnancies were collected for experiments with cultured villous explants (participant characteristics are shown in Table 3.2). All placentas were collected within 30 minutes of delivery.

Table 3.1 Participant demographics of FGR and AGA pregnancies.

Characteristic	AGA controls (n=14)	FGR (n=14)	P value
Birthweight (g)	3417.0±625.0(2916-3930)	2320±795.0 (717-2710)	<0.0001
Birthweight percentile	49.1±18.5 (21.2-79.6)	1.8±1.7 (0-4.5)	<0.0001
Fetal sex (female/male)	8/6	9/5	NS
Gestational age at delivery (weeks)	38.7±0.9 (37.0-40.5)	36.3±2.9 (30.3-39.4)	0.006
Ethnicity			
White British	11 (78.6%)	10 (71.4%)	
Others	3 (21.4%)	4 (28.6%)	
Maternal age (years)	33.9±4.4 (22.0-39.0)	32.3.7±5.0 (20.0-39.0)	NS
Gravidity	2 ± 1 (1-4)	3 ±1 (2-6)	NS
Parity	1 ± 1 (0-3)	2 ±1 (0-4)	NS
Nulliparous (%)	5 (35.7%)	2 (14.3%)	0.03
BMI (kg/m^2)	27.6±5.1 (21-42)	25.9±5.5 (18-36)	NS

FGR, fetal growth restriction; AGA, appropriate for gestational age. Continuous data are shown as mean ± SD or median ± IQR with range, NS, no significant, BMI, body mass index.

Table 3.2 Participant demographics of placental tissue used for cultured explants

Characteristic	Healthy pregnancies (n=15)
Birthweight (g)	3480.4±446.6(3022-4304)
Birth centile	56.1±29.1(26.9-98.4)
Fetal sex (female/male)	5/10
Gestational age at delivery (weeks)	39.2±0.3 (39.0-40.0)
Maternal age (years)	33.9±4.8 (25.0-39.0)
Parity	1 ± 1 (0-2)
Gravidity	3 ± 1 (1-4)
Nulliparous pregnant (%)	1 (6.7%)
Ethnicity	
White British	6 (40.0%)
Black African	4 (26.7%)
Pakistani	2 (13.3%)
Croatian	2 (13.3%)
Others	1 (6.7%)
BMI (kg/m^2)	24.4±4.0 (19.9-28.08)

Continuous data are as mean ± SD or median ± IQR, BMI = body mass index.

3.2.2 Placental explant culture

Villous explants were prepared as previously described (Heazell et al., 2008). Villous tissues (n=8) were sampled from six random sites within the placenta, then were further dissected into 2mm^3 placental explants, and cultured in 1.5ml culture medium (10% CMRL-1066 culture medium (Gibco, UK) supplemented with penicillin (100IU/ml), streptomycin (100µg/ml), L-glutamine (100mg/l), insulin (0.1mg/l), hydrocortisone (0.1mg/l), retinol acetate (0.1mg/l) and 10% fetal calf serum (Gibco, UK). Explants were cultured on Netwells (Corning Inc, NY, USA) at 21% O_2 (5% CO_2, 95% air at 37°C) for 24 hours then transferred to different conditions for up to 4 days, with media changes every 24 hours, in 21% O_2, 1% O_2 or treated with cobalt chloride ($CoCl_2$; 200µM)- a drug that mimics hypoxia *in vitro* by increasing the expression of HIF-1alpha (Manuelpillai et al., 2003). Conditioned culture media was collected at 48, 72 and 96 hours; tissue was collected at 96 hours and processed for RNA, protein, or immunohistochemistry analysis.

3.2.3 Treatment with GSK5182 and DY131

Villous explants from uncomplicated pregnancies (n=7) were maintained in 1.5ml culture medium, composed 1:1 of Dulbecco's modified Eagle medium (DMEM; Gibco, UK)/Ham's F12 (Gibco, UK) supplemented with penicillin (0.6mg/l), streptomycin (100µg/ml), L-glutamine (0.292g/l), and 10% fetal calf serum (Gibco, UK) at 21% O_2 (5% CO_2, 95% air at 37°C) for 24 hours of culture, as previously described (Beards et al., 2017). Culture media was replenished and half of the explants were transferred to the hypoxic incubator (1% O_2, 5%CO_2 at 37°C; 24h), whilst the other half remained at 21% O_2 for a further 24 hours. Media was then replaced with vehicle (0.75% (v/v) DMSO), GSK5182 (20µM or 50µM) or DY131 (20µM or 50µM) and cultured at 21% O_2 or 1% O_2 for an additional 48 hours without changing culture medium. The villous explants and conditioned culture medium were harvested at day 4 of culture.

The method used to transfect villous explants with *ESRRG* siRNAs (Dharmacon, UK), is described in the section 2.2.4 and 2.2.5. BeWo cells (choriocarcinoma cells; ATCC, CCL-98) were also transfected with *ESRRG* siRNAs; the details of BeWo cell culture and siRNA transfection are described in section 2.3.

3.2.4 mRNA extraction and RT-PCR

Prior to mRNA extraction, villous explants were placed in RNAlater overnight and then stored at -80°C. Cultured villous explants or fresh placental tissue was homogenised (using SHM1, UK) and a miRNeasy mini kit (QIAGEN, Germany) was used to extract the total RNA, according to manufacturer's instructions. An AffinityScript cDNA synthesis kit was used for reverse transcription of mRNA to cDNA, following the manufacturer's instructions (Agilent technologies, UK). The PCR primer sequences (Eurofins, UK) for *HIF-1A*, *ESRRG*, its downstream genes and *RPLP0* (60S acidic ribosomal protein P0, house-keeping gene) are listed in Supplementary Table 3.1. Because the mRNA expression of *RPLP0* was stable in human placental tissue (Zhu et al., 2018a), it was used as an internal control. The generated cDNA was amplified by PCR by using a powerup SYBR Green kit (Thermo Fisher Scientific, USA) in the Applied Biosystems Step-one system (Thermo Fisher Scientific, USA). The fold expression was calculated by the $2^{-\triangle\triangle CT}$ method.

3.2.5 Protein preparation and western blotting

Protein from villous explants or placental tissue were extracted using RIPA buffer supplemented with a protease inhibitor complex (1% v/v), phosphatase inhibitor II (1% v/v) and phosphatase inhibitor III (1% v/v). A BCA protein assay (Thermo Fisher Scientific, USA) was used to quantify the protein concentration in the placental lysates.

30µg of protein from fresh placental tissue supernatants or 120µg from placental explant supernatants was loaded into a 10% Sodium-Dodecyl Sulphate polyacrylamide (SDS-PAGE) gel. The gel was run at 120V for 80 minutes, then was transferred to a PVDF membrane (Millipore, Sigma, UK) at 120V for 80 minutes. After blocking in 3% (w/v) milk (Marvel, UK) in PBS for 1 hour at room temperature, the membrane was incubated with an anti-ESRRG (ab128930, monoclonal antibody, 0.12µg/ml, Abcam, UK) primary antibody at 4°C overnight. Beta-tubulin (ab6046, polyclonal antibody, 0.1µg/ml, Abcam, UK) or Beta-actin (20536-1-AP, polyclonal antibody, 0.09µg/ml, Proteintech, UK) was used as the housekeeping protein. After washing, the PVDF membrane was incubated with a fluorescently-conjugated secondary antibody (IRDye green, 0.05µg/ml, LI-COR Bioscience, USA) at room temperature for 1 hour. Antibody

binding and band intensity was quantified using a Licor Odyssey (LI-COR Bioscience, USA) and Image studio lite software (Image studio lite version 5.2, LI-COR Bioscience, USA). The target protein expression was normalised to house-keeping protein.

3.2.6 Analysis of human chorionic gonadotropin (hCG) secretion and lactate dehydrogenase (LDH)

hCG was used as a marker of cytotrophoblast differentiation. hCG in explant-conditioned culture medium was measured by ELISA (DRG Diagnostics, Marburg, Germany). The villous explants, collected at day 4 were lysed in 0.3M NaOH to provide a value for total protein content, measured using a BioRad protein assay (Bio-Rad Laboratories, Hempstead, UK). hCG secretion was expressed as mIU/ml/h/mg protein. Lactate dehydrogenase (LDH) is an enzyme for conversion of lactate to pyruvate in the live cells. It is released from the necrotic cells, so is considered as a marker for cellular viability (Markert, 1984). LDH levels in explant-conditioned culture medium were measured by a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) based on the manufacturer's instructions. LDH release was expressed as absorbance units/mg protein/h.

3.2.7 Immunohistochemical staining

5µm sections from cultured villous explants or freshly collected placental tissues were transferred onto the slides pre-coated with poly-L-lysine. After dewaxing and dehydration, the slides were treated for antigen retrieval by microwave boiling for 5 minutes twice at full power (800w) in 0.01M citrate buffer (PH 6.0) and further incubated with 3% (v/v) hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Slides were incubated with the non-immune block (10% goat serum (v/v) and 2 % human serum (v/v) in 0.1% TBST (TBS-Tween-20) for 30 minutes at room temperature. Sections were incubated with a monoclonal antibody against HIF-1alpha (Abcam, ab51608, 10µg/ml), a polyclonal antibody against ESRRG (Abcam, ab215947, 10µg/ml), a monoclonal antibody against Ki67 (Dako, 0.174µg/ml), or a monoclonal antibody against M30 (Roche, 0.132µg/ml) overnight at 4°C. Matched concentrations of isotype-specific non-immune rabbit or mouse IgG were used as a negative control. After washing, biotin conjugated goat anti-mouse or anti-rabbit

antibodies (Dako-Cytomation, 3.85µg/ml) were applied and incubated for 30 minutes at room temperature as a secondary antibody. After the incubation with avidin-peroxidase (5µg/ml) for 30 minutes, chromogenic substrate diaminobenzidine (DAB; Sigma-Aldrich, UK) was applied to the sections for between 2-10 minutes. Colour development was monitored under a microscope. Harris's hematoxylin (Sigma-Aldrich, UK) was used to counterstain all slides for 5 minutes, then was further differentiated with acid alcohol for 2 seconds. All villous explants and placental tissues were stained in the same batch for comparison; no immunoactivity was observed on the negative controls.

3.2.8 Statistical analysis

All data are presented as the mean ± standard deviation (SD) (normally distributed) or median ± interquartile range (IQR) (non-normally distributed); the Shapiro-Wilk test was used to determine whether the data were normally distributed. GraphPad Prism version 8.0.1 (GraphPad Software, USA) was used to undertake the statistical analysis. Data were assessed using an unpaired t test or one-way ANOVA for normally distributed data or using a Mann-Whitney U Test or Kruskal-Wallis, followed by a Friedman multiple comparison test for non-parametric data. QuPath (version 0.2.3, developed by University of Edinburgh (Bankhead et al., 2017)), was used to quantify the immunostaining. DAB staining was quantified to measure the extent of Ki67 and M30 staining; this was expressed as a percentage of the total number of cells. A P value <0.05 was considered as indicative of statistical significance.

3.3 Results

3.3.1 Reduced expression of *ESRRG* and its downstream genes in FGR placentas

The mean *ESRRG* mRNA expression level in the FGR group was 34.1% lower than AGA group (Figure 3.1A) (P=0.005) and the mean ESRRG protein level in FGR placentas was significantly reduced, by 53.5%, compared with AGA placentas (Figures 3.1B and 3.1C, P=0.004). Immunostaining for protein expression of ESRRG (Figure 3.1D) in AGA placentas (b) and FGR placentas (c) suggest the expression of ESRRG protein is mainly localised within the syncytiotrophoblast and stromal cells. The mRNA expression level of four genes downstream of *ESRRG*, *HSD17B1*, *HSD11B2*, *CYP19A1.1*, and *PLAC1* were also assessed in FGR and AGA placentas (Figure 3.1E). The median mRNA level of *HSD17B1* and *PLAC1* was not significantly reduced in FGR compared to that observed in AGA placentas (Figure 3.1E, (a) and (d)); however, the median mRNA levels of *HSD11B2* and *CYP191.1* were significantly decreased by 29% and 25% in FGR placentas respectively, when compared to AGA placentas (Figure 3.1E, (b) and (c), P<0.05).

3.3.2 Hypoxia reduces *ESRRG* signalling pathway in cultured villous explants

Changes in the mRNA and protein expression of ESRRG and its four downstream genes in villous explants cultured in 21% O_2 over 96h are shown in Supplementary Figure 3.1 and Supplementary Figure 3.2. Compared to the fresh placental tissue, the median mRNA levels of *ESRRG* were decreased in the cultured placental explants (Supplementary Figure 3.1A) and ESRRG protein expression was dramatically decreased in the cultured explants from 24 hours to 96 hours (Supplementary Figure 3.1B, C, E and F). The median mRNA levels of ESRRG's downstream genes, *CYP191.1*, *HSD17B1*, *HSD11B2*, and *PLAC1*, were lower in explants cultured for 24 - 96 hours than in fresh placental tissue (Supplementary Figure 3.2).

Compared to explants cultured in 21% O_2, mRNA expression of *HIF-1A* was increased in the explants treated with $CoCl_2$ (P=0.01), but was unchanged in the explants cultured in 1% O_2 (Figure 3.2A). The protein staining results indicated HIF-1alpha immunostaining was rarely observed in the explants cultured in 21% O_2 (Figure 3.2B

(b)) but was present in the nuclei of the cytotrophoblasts and stromal cells in explants treated with $CoCl_2$ (Figure 3.2B (c)) or cultured in 1% O_2 (Figure 3.2B (d)). Median *ESRRG* mRNA expression was significantly decreased by 51.6% in the explants maintained in 1% O_2 (Figure 3.2C; P=0.005) and decreased by 53.7% in placental villous explants exposed to $CoCl_2$ (Figure 3.2C; P=0.04). Whilst the results of western blotting showed that total tissue ESRRG protein expression was unchanged across treatment groups (Figure 3.2D and 3.2E), immunohistochemical staining highlighted that compared to explants cultured at 21% O_2 (Figure 3.2F (b)), ESRRG protein expression was reduced in the stroma and trophoblast of explants exposed to $CoCl_2$ (Figure 3.2F (c)) or 1% O_2 (Figure 3.2F (d)).

The median level of *HSD17B1* mRNA expression was reduced by 39.1% after exposure to $CoCl_2$ (Figure 3.2G, (a), P=0.02) and by 49.8% after exposure to 1% O_2 (Figure 3.2G, (a), P=0.0009). Compared to explants cultured at 21% O_2 the median mRNA level of *HSD11B2* was decreased by 3-fold in explants cultured at 1% O_2 (Figure 3.2G, (b), P=0.01) or treated with $CoCl_2$ (Figure 3.2G, (b), P=0.002). Moreover, the median mRNA level of *CYP191.1* was decreased by 37.4% after culture in 1% O_2 (Figure 3.2G, (c), P=0.005) and by 41.6% in explants exposed to $CoCl_2$ (Figure 3.2G, (c), P=0.005). *PLAC1* mRNA was also reduction in explants exposed to $CoCl_2$ (86.5%; P=0.0004, Figure 3.2G, (d)) and in explants exposed to 1% O_2 (72.0%; P=0.04, Figure 3.2G, (d)), compared to those cultured at 21% O_2.

The relative expression of ESRRG and its downstream genes in explants which were cultured at 6% O_2 versus 21% O_2 are shown in Supplementary Figure 3.3. There is no difference in the mRNA (Supplementary Figure 3.3A) and protein expression of ESRRG in the explants cultured under 21% O_2 or 6% O_2 (Supplementary Figure 3.3B-D). The mRNA levels of *ESRRG*'s downstream genes, including *HSD17B1*, *CYP191.1*, *HSD11B2*, *PLAC1* (Supplementary Figure 3.3E-H) were comparable in the explants cultured in 21% O_2 or 6% O_2.

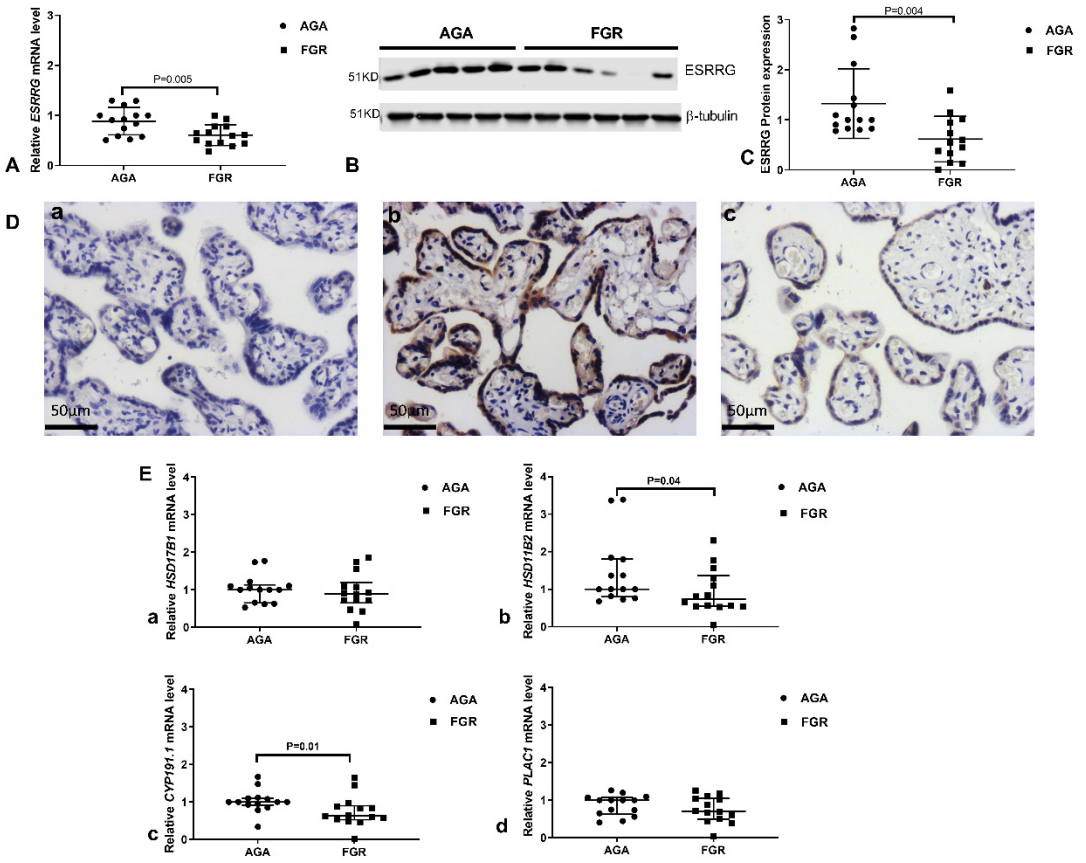

Figure 3.1 The expression of ESRRG and its downstream genes in placentas from AGA pregnancies and those with FGR.

[A] mRNA expression of *ESRRG*. [B] Representative western blot of ESRRG and house-keeping gene beta-tubulin protein expression. [C] Quantification of band density. [D] ESRRG protein staining in both AGA (b) and FGR placentas(c); (a) negative control with non-immune rabbit IgG without primary antibody. [E] the mRNA levels of *ESRRG*'s downstream genes: [a] *HSD17B1*, [b] *HSD11B2*, [c] *CYP191.1*, [d] *PLAC1*. FGR: fetal growth restriction; AGA: appropriate for gestational age; *ESRRG*: oestrogen related receptor gamma; *HSD17B1*: hydroxysteroid 17-beta dehydrogenase 1; *HSD11B2*: hydroxysteroid 11-beta dehydrogenase 2; *CYP191.1*: cytochrome P-450; *PLAC1*: placenta-specific 1. [A] and [C] mean +/- SD, statistical significance was assessed by unpaired t test. [E] median +/- interquartile range (IQR), Mann-Whitney test was used.

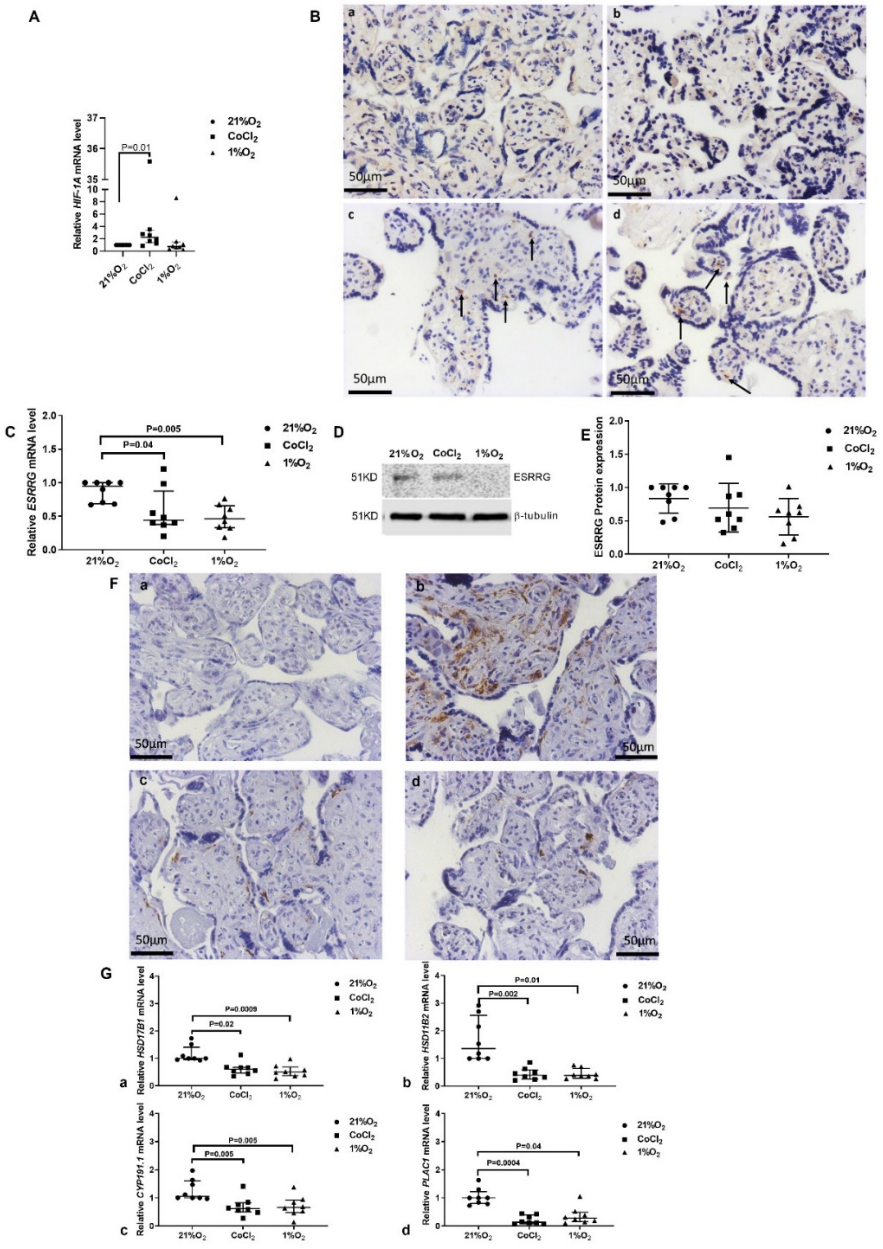

Figure 3.2 mRNA and protein expression of HIF-1alpha, ESRRG and its downstream genes in explants from placentas cultured in 21% O_2, 1% O_2 and following treatment with cobalt chloride ($CoCl_2$).

[A] mRNA expression of *HIF-1A*. [B] Representative HIF-1alpha protein staining images of explants cultured in 21% O_2 (b), treated with $CoCl_2$ (c), or cultured in 1% O_2 (d); negative control was the placental explants applied with non-immune mouse IgG substituting HIF-1alpha primary antibody (a). [C] mRNA levels of *ESRRG*. [D] Representative blot of ESRRG and beta-tubulin (house-keeping gene). [E] Quantification of band density. [F] Representative ESRRG immunostaining images of

placental explants cultured under 21% O_2 (b), $CoCl_2$ treatment (c), or 1% O_2 (d); [a] negative control with non-immune rabbit IgG replacing the primary antibody (a). Scale bar = 50µm. [G] mRNA expression of *ESRRG*'s downstream genes: [a] *HSD17B1*, [b] *HSD11B2*, [c] *CYP191.1*, [d] *PLAC1*. Statistical significance was assessed by Friedman test; median +/- IQR.

3.3.3 GSK5182 and DY131 rescued hypoxia-mediated alterations in *ESRRG* and its downstream genes

The results of transfection of BeWo cells and cultured villous explants with *ESRRG* siRNAs are shown in Supplementary Figures 3.4-3.6. In BeWo cells, *ESRRG* mRNA expression did not change when the cells were transfected with *ESRRG* siRNAs (20nM to 200nM) for 48 hours of culture, compared to untreated control explants (Supplementary Figure 3.4A). In addition, *ESRRG* siRNAs (20nM) did not reduce *ESRRG* mRNA expression in BeWo cells over a 24-72h exposure (Supplementary Figure 3.4B). Meanwhile, transfecting BeWo cells with 40nM *ESRRG* siRNAs did not silence *ESRRG* mRNA expression for 48 hours of culture (Supplementary Figure 3.4C).

In cultured term placental explants transfected with *ESRRG* siRNAs (13.3nM to 66.7nM), the median mRNA expression level of *ESRRG* was decreased in explants transfected with 13.3nM or 26.7nM of *ESRRG* siRNAs, but this decrease was not statistically significant (Supplementary Figure 3.5A). Although reduced syncytial immunostaining of ESRRG was observed in explants transfected with *ESRRG* siRNAs (13.3nM or 26.7nM) (Supplementary Figure 3.5C), the mRNA expression of *ESRRG* (Supplementary Figure 3.5B) and its downstream genes (Supplementary Figure 3.5D and 3.5E) was comparable across treatment groups. There was no alteration in either ESRRG mRNA (Supplementary Figure 3.6A) or protein expression in the explants incubated with different concentrations of *ESRRG* siRNAs or the non-targeting control siRNA (10nM to 80nM) (Supplementary Figure 3.6B-G).

As siRNA-mediated *ESRRG* silencing was unsuccessful in BeWo cells and placental explants, we sought to manipulate *ESRRG* signaling by applying the *ESRRG* agonist DY131, or the inverse agonist GSK5182 to term placental explants. Treatment of explants cultured at 21% O_2 with either GSK5182 or DY131 did not alter the mRNA expression of *ESRRG*, *HSD17B1*, *HSD11B2*, and *PLAC1* (Figure 3.3A, Figure 3.4A, 3.4B, and 3.4D). GSK5182 (50μM) and DY131 (20μM) increased mRNA expression of *CYP191.1* (Figure 3.4C, $P<0.05$).

Under hypoxic conditions (1% O_2), GSK5182 and DY131 reversed the low expression of *ESRRG*; GSK5182 (20μM) significantly increased the mRNA level of *ESRRG* by 1.5-

fold, compared to DMSO controls (Figure 3.3A, P=0.04), and GSK5182 (50μM) and DY131 (50μM) increased the protein expression of ESRRG by 1.9-fold and 1.6-fold, respectively (Figure 3.3B, and Figure 3.3C, P<0.05). For *ESRRG*'s downstream genes, GSK5182 (20μM) increased the mRNA expression of *HSD17B1*, *CYP191.1*, and *PLAC1* in 1% O_2 (Figure 3.4A, 3.4C, and 3.4D). Meanwhile, DY131 (50μM) increased mRNA expression of *HSD11B2* and *CYP191.1* by 1.5-fold and 2.3-fold, respectively (Figure 3.4B and 3.4C).

3.3.4 DY131 increases the number of cells in cycle and reduces apoptosis induced by hypoxia

Compared to the villous explants cultured in 21% O_2, the number of cells in cycle was significantly decreased, and apoptotic cell death was significantly increased in villous explants cultured in 1% O_2 or treated with $CoCl_2$ (Supplementary Figure 3.7A and 3.7B). In contrast, the number of Ki-67 and M30-positive cells were similar in explants cultured at 21% O_2 and 6% O_2 (Supplementary Figure 3.8). Application of DY131 (50μM) increased the number of cells in cycle in hypoxic conditions (Figure 3.5A, and Supplementary Figure 3.9, P<0.05), and hypoxia induced apoptotic cell death was significantly decreased in villous explants treated with DY131 (20μM and 50μM) (Figure 3.5B, and Supplementary Figure 3.10, P<0.05).

3.3.5 DY131 reduces hypoxia induced necrosis of villous explants

The mean hCG levels at day 4 of culture were 55.3% or 50% lower following exposure to 1% O_2 or treated with $CoCl_2$ (P=0.02) (Supplementary Figure 3.11A and B). hCG secretion was significantly decreased in explants cultured in 6% O_2 when compared to explants cultured in 21% O_2 for 96 hours (Supplementary Figure 3.12A and B). The total hCG secretion was significantly higher in conditioned culture medium of GSK5182 (20μM) treated explants compared to controls cultured at 21% O_2, but not in hypoxia (Figure 3.6A).

LDH release was comparable between explants cultured at 21% O_2 and 6% O_2 (Supplementary Figure 3.12C and D). From day 2 to day 4, there was limited LDH release from the explants cultured in 21% O_2 (Supplementary Figure 3.11C), however at day 4 of culture in 1% O_2, the mean LDH release from explants maintained was

increased (P=0.02, Supplementary Figure 3.11D) demonstrating a reduction in tissue viability and cellular integrity in this group. This increase in LDH levels was significantly decreased in tissue cultured in DY131 (20μM) at 1% O_2 (Figure 3.6B, P=0.01). A reduction in LDH levels was also seen in explants cultured at 21% O_2 that were treated with DY131.

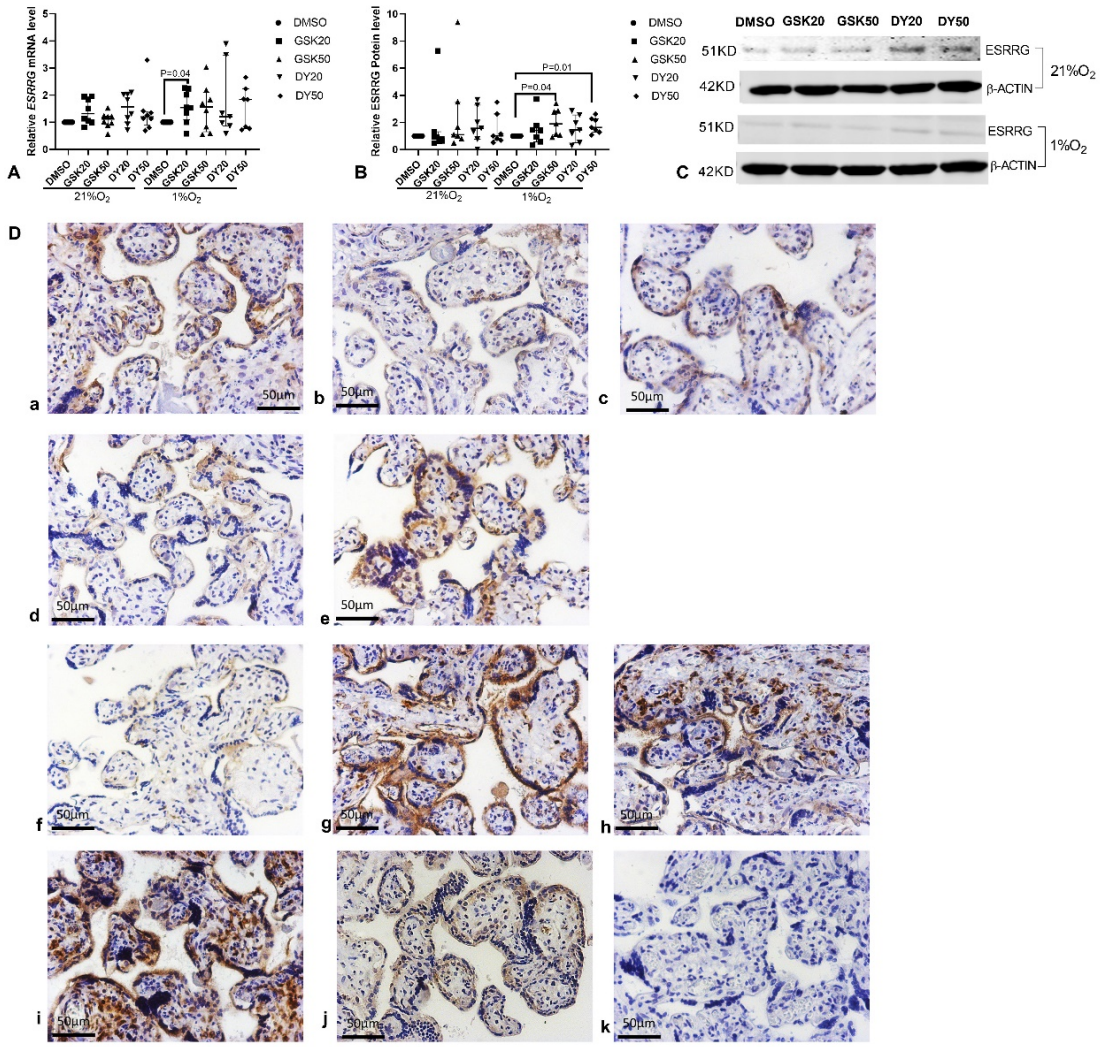

Figure 3.3 The expression of ESRRG in AGA placental explants cultured in 21% O_2 or 1% O_2 with the treatment of GSK5182 or DY131.

[A] mRNA expression of *ESRRG*; [B] Protein quantification of band density from Western blotting. [C] Representative Western blot of ESRRG and house-keeping gene, beta-actin. [D] Representative images of immunochemistry staining for ESRRG in the explants cultured at 21% O_2 (a-e) or 1% O_2 (f-j). (k) Negative control with non-immune rabbit IgG. Villous explants were treated with 0.75% DMSO (a, f), GSK5182 (20μM) (b, g), GSK5182 (50μM) (c, h), DY131 (20μM) (d, i) or DY131 (50μM) (e, j). Mark=50μM. DMSO, Dimethyl sulfoxide; GSK20, GSK5182 (20μM); GSK50, GSK5182 (50μM); DY20, DY131 (20μM); DY50, DY131 (50μM). Median +/- IQR; One sample Wilcoxon test was used to examine statistical significance.

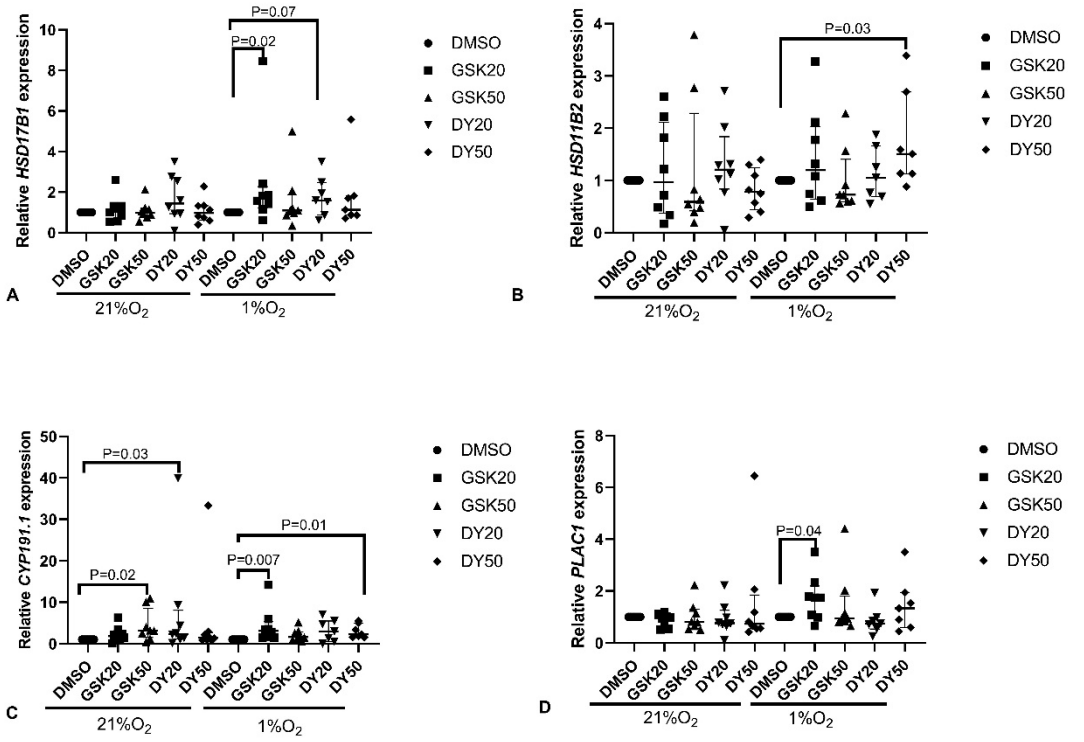

Figure 3.4 mRNA expression of downstream genes of *ESRRG* in the villous explants with GSK5182 or DY131 treatment in different oxygen concentrations.

[A] *HSD17B1* [B] *HSD11B2* [C] *CYP191.1*, [D] *PLAC1.* DMSO, Dimethyl sulfoxide; GSK20, GSK5182 (20μM); GSK50, GSK5182 (50μM); DY20, DY131 (20μM); DY50, DY131 (50μM). Median +/- IQR; One sample Wilcoxon test was used to examine statistical significance.

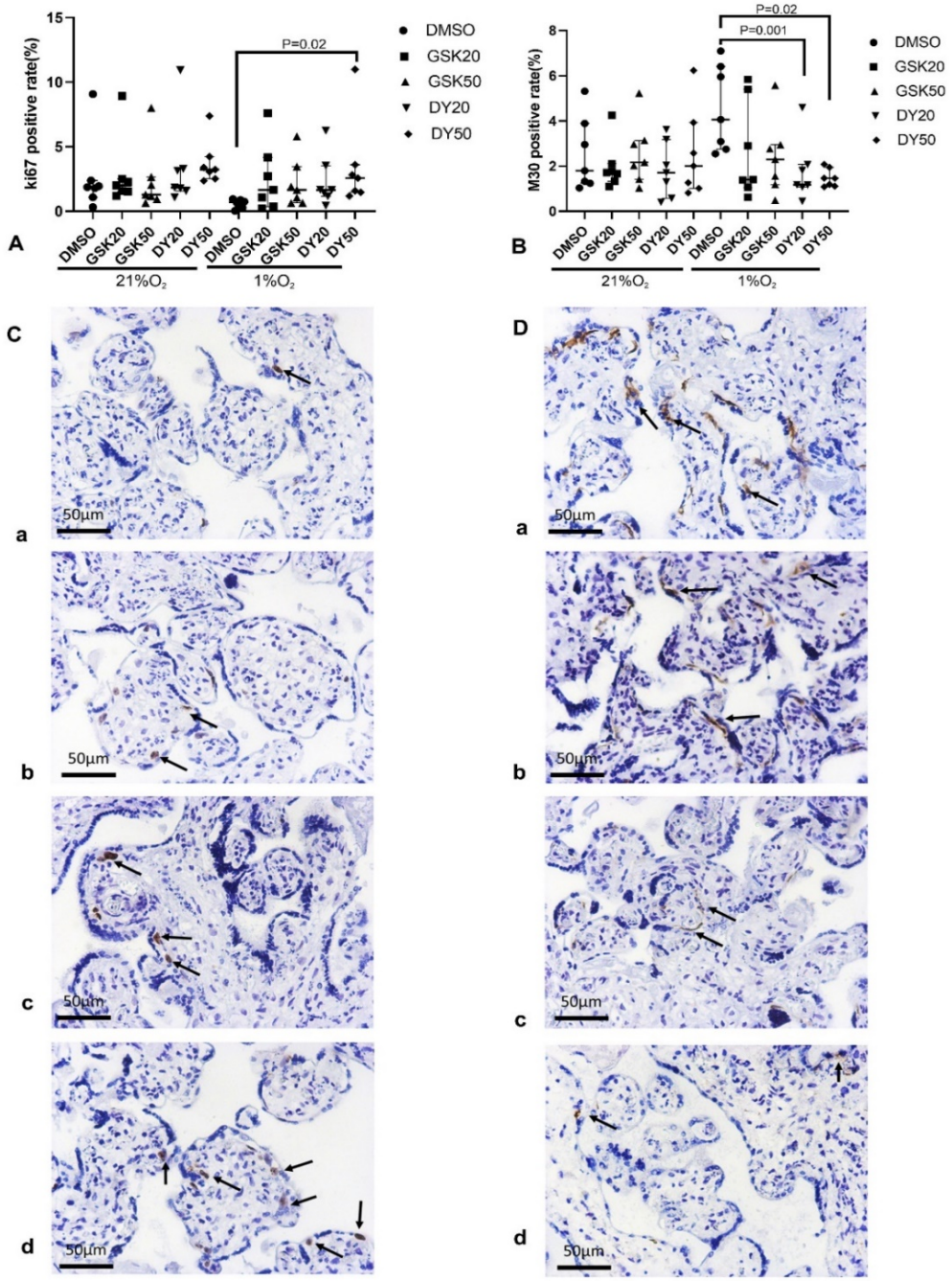

Figure 3.5 The Ki-67 and M30 staining in cultured villous explants following treatment with GSK5182 and DY131.

The quantification of Ki-67 [A] and M30 [B] staining. [C] and [D] is the representative images of immunostaining for Ki-67 [C] and M30 [D] in the villous explants cultured at 1% O_2. [a] 0.75% DMSO; [b] GSK5182 (20μM); [c] DY131 (20μM); [d] DY131(50μM).; Median +/- IQR; Friedman test.

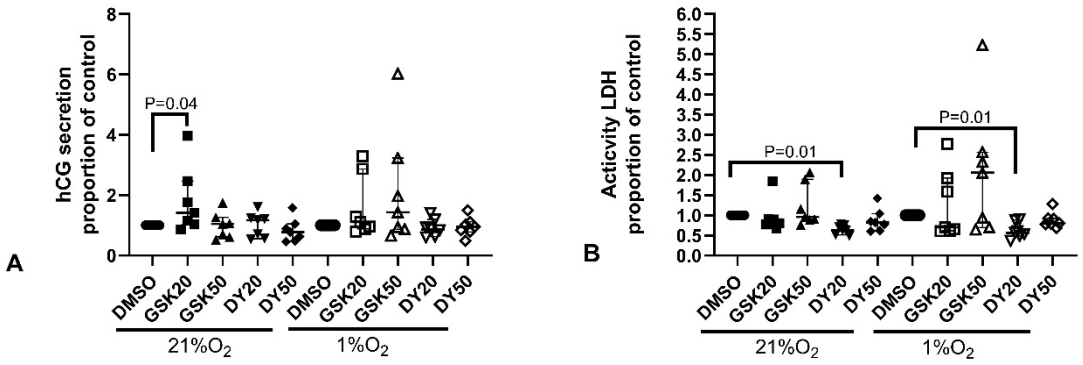

Figure 3.6 hCG secretion and LDH release in conditioned culture media.

Figure 3.6A, The secretion of total hCG. Figure 3.6B The LDH levels in the culture medium. LDH: lactate dehydrogenase; hCG: human chorionic gonadotropin. Median +/- IQR; One sample Wilcoxon test was used to examine statistical significance.

3.4 Discussion

This study demonstrates that both mRNA and protein expression of ESRRG and the mRNA expression of its downstream genes are decreased in human FGR placentas, and this can be reproduced by culturing healthy placental explants in 1% O_2 or exposing them to $CoCl_2$. An agonist of *ESRRG*, DY131, rescued the abnormal cell turnover induced by hypoxia, by modulating *ESRRG* signalling. These findings suggest that the *ESRRG* pathway is dysregulated in FGR and may mediate some of the downstream effects of placental hypoxia, a key contributor to placental dysfunction (Figure 3.7).

3.4.1 Reduced *ESRRG* signalling in human placentas is related to FGR

Our assessment of AGA and FGR placentas confirms that the mRNA and protein level of ESRRG is reduced in FGR placentas, which is consistent with previous studies (Poidatz et al., 2015a, Zhu et al., 2018a). These studies used placental samples from southern Chinese and French populations which defined FGR as estimated fetal weight or birth weight less than 10th percentile. Most of our samples were from Caucasians in the UK, and a more stringent definition of FGR (IBR less than 5th percentile) was applied. The consistent nature of this finding in three different populations suggests that a reduction in *ESRRG* expression is observed in FGR.

To further investigate the *ESRRG* signaling pathway, we quantified expression of four genes downstream from *ESRRG*. We found the mRNA expression of *HSD11B2* and *CYP19A1.1* was significantly decreased in FGR placentas. *CYP191.1* encodes the aromatase P450 which plays a role in the conversion of C19 steroid precursors into oestrogen. Data regarding *CYP191.1* expression in FGR placentas are inconsistent. One previous study of FGR placentas (IBR below 10th centile) found increased expression of *CYP191.1* (Anelli et al., 2019). It is possible that the reduced expression level of *CYP19A1.1* observed in our study might be related to the severity of FGR. *HSD11B2*, encodes an enzyme related to the conversion of active cortisol to inactive cortisone which is expressed in villous syncytiotrophoblast; placental *HSD11B2* level is correlated with fetal weight and postnatal growth velocity (McTernan et al., 2001, Struwe et al., 2007, Zhu et al., 2018b). Similar to a previous study (Struwe et al., 2007), our results also revealed reduced mRNA expression of *HSD11B2* in FGR placentas.

HSD17B1 encodes a steroidogenic enzyme responsible for converting estrone to 17 beta-oestradiol. Previous reports have found *HSD17B1* is decreased in FGR placentas (IBR less than 10th centile) and activated by *ESRRG* in the HTR-8/Svneo cell line (Zhu et al., 2018a). *PLAC1* was mainly expressed in syncytiotrophoblast and increased in the FGR placentas. However, our results did not show a statistical difference in the mRNA expression of *HSD17B1* and *PLAC1* in FGR placentas and this may be due to the difference in race and the definition of FGR placentas among these studies.

3.4.2 The *ESRRG* signalling pathway is hypoxia-responsive

We used a four-day explant culture at 1% O_2 or treatment with $CoCl_2$ to mimic the placental hypoxia observed in FGR. This model has previously been well characterized and reproduces aspects of FGR, including reduced proliferation and increased apoptosis (Heazell et al., 2008, Heazell et al., 2011, Diaz et al., 2016). Both hypoxia and $CoCl_2$ (a chemical hypoxia mimic) can stabilize HIF-1alpha (encoded by *HIF-1A*) (Castro-Parodi et al., 2013, Francois et al., 2017), which is also elevated in FGR placentas (Tal et al., 2010, Stubert et al., 2012). HIF-1 is a heterodimer composed of the HIF-1alpha subunit and the HIF-1beta subunit (Wang et al., 1995). HIF-1alpha is degraded under normoxic conditions, but rapidly accumulates in hypoxia, where it can combine with HIF-1beta to transcriptionally modulate the expression of HIF-1 responsive downstream genes (Wang et al., 1995, Salceda and Caro, 1997). As we expected, mRNA expression of *HIF-1A* was significantly increased in explants treated with $CoCl_2$. We also observed increased immunostaining of HIF-1alpha protein in explants exposed to $CoCl_2$ or 1% O_2, but not in explants cultured in 21% O_2. However, although the median mRNA expression level of *HIF-1A* was reduced in the explants cultured in 1% O_2, the reduction was not statistically significant. Interestingly, many previous studies also observed decreased *HIF-1A* mRNA expression in hypoxia, while its protein expression was transiently increased in lymphocytes, intestinal epithelial cells, the human lung epithelial A549 cell line, and HMEC-1 cells, a human microvascular endothelial cell line (Thrash-Bingham and Tartof, 1999, Uchida et al., 2004, Bruning et al., 2011, Chamboredon et al., 2011, Cavadas et al., 2015). These data suggest more complex regulation of HIF-1alpha mRNA and protein expression, which may be regulated by other transcription factors such as repressor element 1-silencing

transcription factor or by miRNAs, such as miR-155 (Bruning et al., 2011, Cavadas et al., 2015). Kumar et al. demonstrated that high levels of HIF-1alpha protein expression can downregulate both ESRRG mRNA and protein expression in primary second trimester cytotrophoblast cultured in 2% O_2 (Kumar and Mendelson, 2011). We also observed an increased protein expression of HIF-1alpha in explants treated with $CoCl_2$ or cultured in 1% O_2, which correlated with reduced expression of ESRRG expression in our term explant model.

Both treatments ($CoCl_2$ and 1% O_2) reduced hCG secretion and LDH release, compared to control explants cultured at 21% O_2, suggesting these pathways might be regulated by the O_2-sensitive transcription factor, HIF-1alpha. In common with previously reported findings in second trimester placental tissue, there was lower ESRRG expression at both the mRNA and protein level (Kumar and Mendelson, 2011). As ESRRG localizes to the syncytiotrophoblast layer and stromal cells in placental explants, one could hypothesise that it is involved in regulating trophoblast function. However, the levels of ESRRG protein measured by Western blotting after four days of culture were much lower than in fresh tissue and were not significantly decreased at 1% O_2. This suggests there also could be other regulators of the signaling pathways involving *ESRRG* in hypoxia; to further explore this pathway, the identification of *ESRRG*'s downstream effectors is needed.

All of the genes downstream of *ESRRG* measured here were reduced following culture in 1% O_2 or following treatment with $CoCl_2$. Previously *CYP191.1* has been validated as downstream effectors of *ESRRG* in primary second trimester cytotrophoblast (Kumar and Mendelson, 2011). Our findings provide further evidence for the regulation of *CYP191.1* by *ESRRG*, as the present study is the first to use human placental tissue. The reduced mRNA level of *CYP19A1.1* in 1% O_2 and $CoCl_2$ group is in agreement with Kumar et al., who investigated second-trimester cytotrophoblast (Kumar and Mendelson, 2011). To our knowledge, our data are the first to suggest that *ESRRG* might mediate the expression of *HSD11B2* in hypoxia. The reduced expression of *HSD11B2* in hypoxia has been established previously *in vitro* and *in vivo* studies of pregnancy (Hardy and Yang, 2002, Cuffe et al., 2014). Homan et al. found a low promoter activity in *HSD11B2* in a primary cytotrophoblast model under hypoxic

conditions (1% O_2) (Homan et al., 2006), suggesting that this may be in response to a lack of an upstream stimulus. As we report low mRNA expression of *ESRRG* and *HSD11B2* in our explant model in both 1% O_2 and $CoCl_2$ groups, it is possible that low levels of *HSD11B2* are mediated by hypoxia-induced reductions in the *ESRRG* signaling pathway.

3.4.3 GSK5182 and DY131 rescue hypoxia induced alterations in the *ESRRG* signalling pathway

We were unsuccessful in using siRNAs to reduce the mRNA and protein expression of ESRRG in either BeWo cells or placental explants. Although we used a pooled *ESRRG* siRNAs containing three different *ESRRG* siRNAs with several concentrations, these failed transfections on the cell lines or explants could be caused by the low transfection efficiency of these siRNAs, insufficient siRNA concentrations or inappropriate transfection protocols we used in this study. In addition, for the explants, this may be because the mRNA and protein expression of ESRRG were significantly reduced in cultured explants compared to fresh placental tissue (Supplementary Figure3.1), making it harder to further reduce the expression of *ESRRG* using siRNAs.

In this study, GSK5182 and DY131 rescued the reduction in *ESRRG* expression, and its downstream genes induced by hypoxia. Interestingly, we observed that GSK5182, a reverse agonist for *ESRRG*, can induce *ESRRG* mRNA and protein expression, as well as that of its downstream genes, in hypoxia. In previous studies using cell lines, GSK5182 was shown to inhibit the transcription of *ESRRG* (Kim et al., 2013, Singh et al., 2015). However, this study used a cultured villous explant model, which had a very low protein expression of *ESRRG* as mentioned above. Therefore, this might suggest GSK5182 could not further reduce the gene expression of *ESRRG,* but instead induced its expression in hypoxia by activating coactivators or other regulators of *ESRRG*. Further studies are needed to uncover this unexpected effect of GSK5182 in placental villous explants.

DY131, an agonist of *ESRRG*, can induce the expression of *ESRRG* in animal models or primary cytotrophoblast (Yu and Forman, 2005, Luo et al., 2014, Poidatz et al., 2015b, Kang et al., 2018). DY131 is a synthesized *ESRRG* ligand as acyl hydrazone derivatives

are known potential ligands for *ESRRG* and DY131 is developed as an acyl phenylhydrazone derivative that can activate the expression of *ESRRG* (Yu and Forman, 2005). Our study suggests that DY131 can also induce the protein expression of *ESRRG* and its downstream genes (*HSD11B2* and *CYP191.1*) in hypoxia, in a placental explant model, which provides further support that both *HSD11B2* and *CYP191.1* are downstream genes of *ESRRG* in the placenta. This restoration of effect supports the hypothesis that *ESRRG* can regulate some of the effects of hypoxia in trophoblast by mediating the downstream genes, *HSD11B2* and *CYP191.1*.

3.4.4 Potential therapeutic efficacy of the *ESRRG*'s agonist DY131 in the placental dysfunction

The reduction in hCG secretion on day 4 of culture in 1% O_2 is consistent with previous reports of this explant model or primary trophoblast (Esterman et al., 1996, Crocker et al., 2004, Heazell et al., 2008, Diaz et al., 2016), which suggests an impaired differentiation of syncytiotrophoblast in hypoxia. Like our previous study (Heazell et al., 2008), hypoxia reduced the number of cells in cycle and increased apoptosis, but the proliferative rate and apoptotic rate in this study are much lower than our previous results which might be related to the tissue variability. GSK5182 and DY131 did not have any effects upon hCG secretion from cultured villous explants in hypoxia, but DY131 treatment reduced the level of cell necrosis, increased the number of cells in cycle and reduced apoptotic cell death in explants cultured under hypoxic conditions. These results suggest that *ESRRG* signalling mediates some of the downstream effects of hypoxia, which are implicated in the pathophysiology of FGR. Two studies have already identified the possibility of using DY131 as a therapeutic intervention in mice (Luo et al., 2014, Ma et al., 2021). Ma et al. measured the toxicity of DY131 (5mg/kg) on the mice and they did not find any obvious side effects of DY131 on the hepatic and renal functions (Ma et al., 2021). Luo et al. applied DY131 (8mg/ml) in pregnant mice from 10.5 to 17.5 days post coitum (dpc) and they observe that DY131 caused the hypertension from 16.5 dpc, but this study did not explore the safety of DY131 on these pregnant mice (Luo et al., 2014). Our observations suggest that DY131 could be investigated as a potential therapeutic agent to treat hypoxia-

induced placental dysfunction. However, further studies are required to assess the safety and efficacy of DY131 in the pregnant animal models.

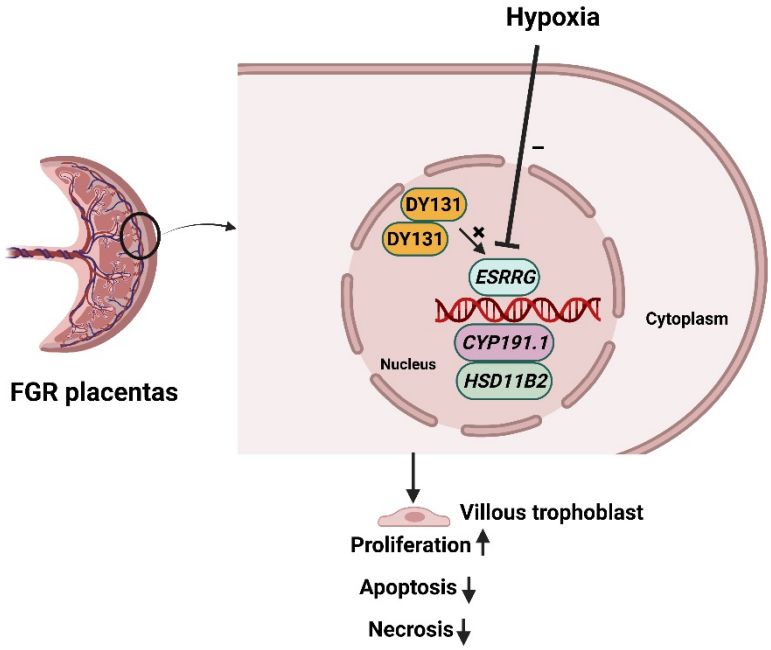

Figure 3.7 The schematic of the ESRRG pathway.

ESRRG: Oestrogen related receptor gamma; *CYP191.1*: Cytochrome P-450; *HSD11B2*: Hydroxysteroid 11-beta dehydrogenase 2.

3.4.5 Strengths and Limitations

This study has further confirmed that *ESRRG* signaling is reduced in placentas from FGR pregnancies, even when applying a more stringent definition of FGR. We firstly explored the relationship between hypoxia-mediated *ESRRG* signalling pathways and cell turnover in a cultured villous explant model, arguably a more physiologically relevant culture model than placental cell lines or isolated primary cells. Additionally, this is the first study to explore whether *ESRRG*'s agonist, DY131, can restore impaired *ESRRG* signalling and rescue the aberrant cell turnover observed in villous explants exposed to hypoxia. This work has identified DY131 as a potential therapeutic for treating pregnancies complicated with FGR, which needs to be further investigated.

Although our results are consistent with previous reports in other populations further studies are needed to confirm whether *ESRRG* is reduced in other populations with FGR. To explore whether *ESRRG* mediated the phenotype seen in FGR, this study used a short-term culture of placental villous explants, but the time period selected was not able to identify the effects of longer-term culture, which should also be explored. In addition, another weakness of this study is the villous explants model because of the low protein expression of ESRRG signalling pathways in this model and the different ESRRG protein localisation between fresh placental tissue and villous explants which may hamper the exploration of these pathways.

3.5 Conclusion

The present study demonstrates that *ESRRG* signaling is dysregulated in FGR. The molecular mechanism underlying the regulatory role of *ESRRG* in FGR appears to be mediated in part through the hypoxia sensitive *ESRRG* signalling pathway. An *ESRRG* agonist, DY131, can increase *ESRRG* signalling and rescue the abnormal cell turnover observed in placental explants cultured under hypoxia. Modulation of this signalling pathway offers a novel therapeutic option for the treatment of FGR.

Author contribution statement

Zhiyong Zou, Lynda K. Harris, Karen Forbes, and Alexander E.P. Heazell conceived and designed the research. Zhiyong Zou conducted the experiments. Alexander E.P. Heazell, Karen Forbes, and Lynda K Harris contributed to the reagents and analytical kits, and Zhiyong Zou analyzed and interpreted the data, drafted the manuscript. All authors read and approved the final manuscript.

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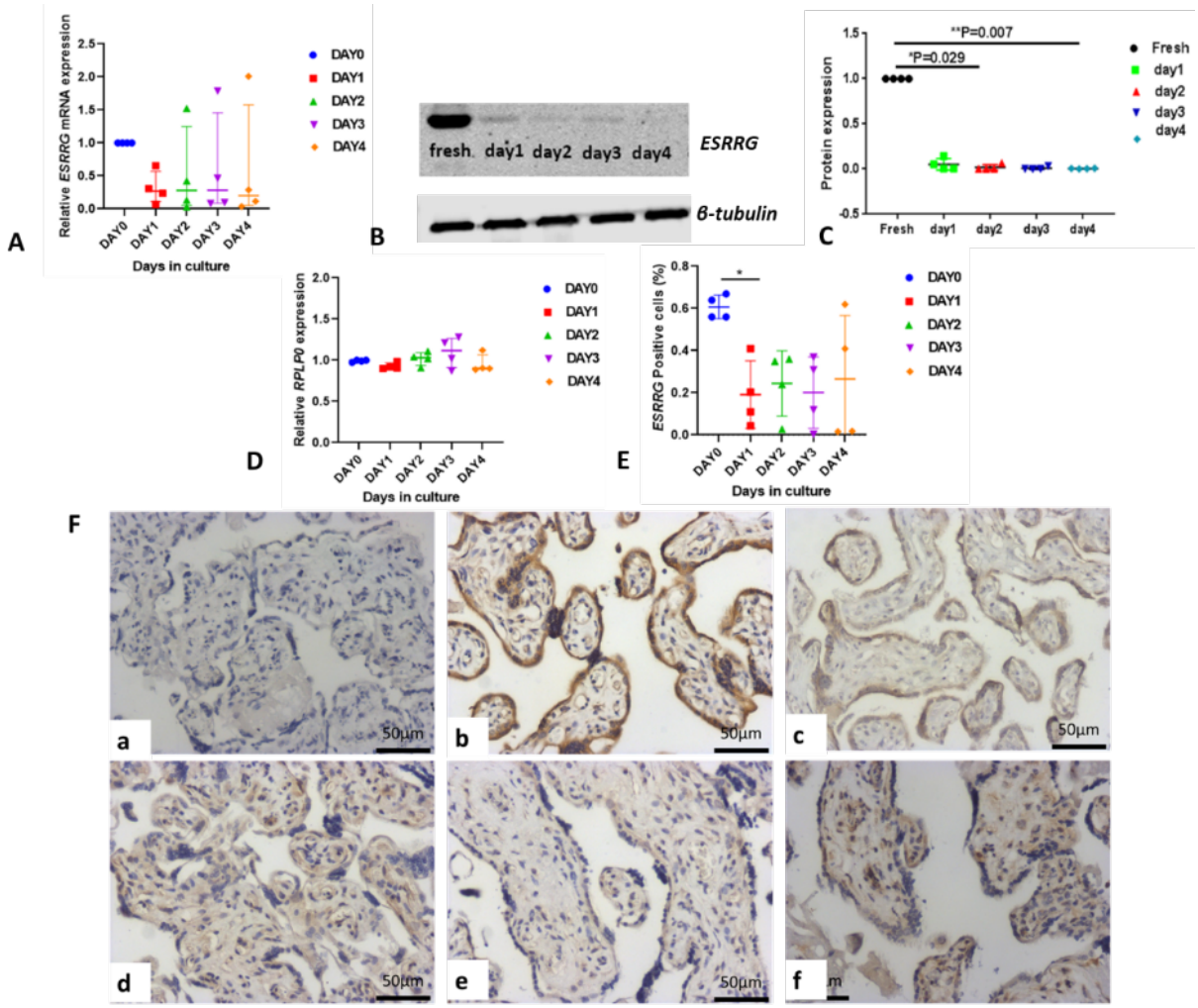
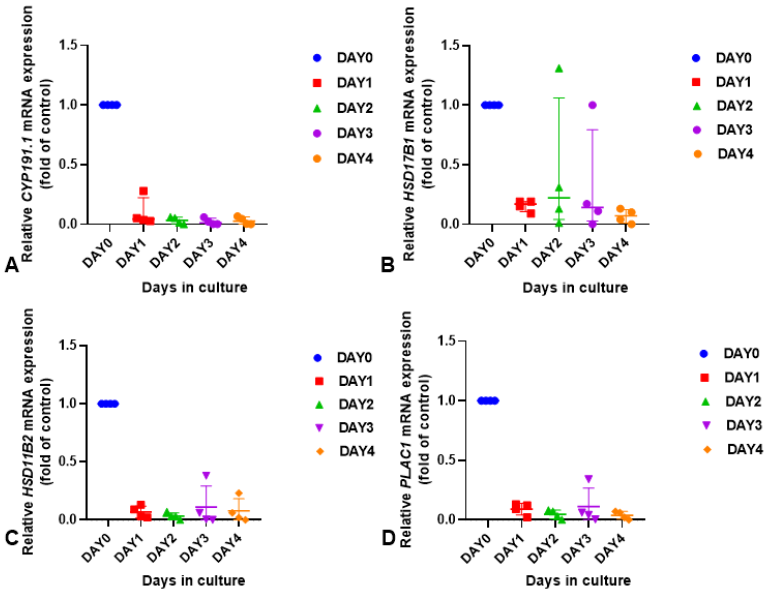


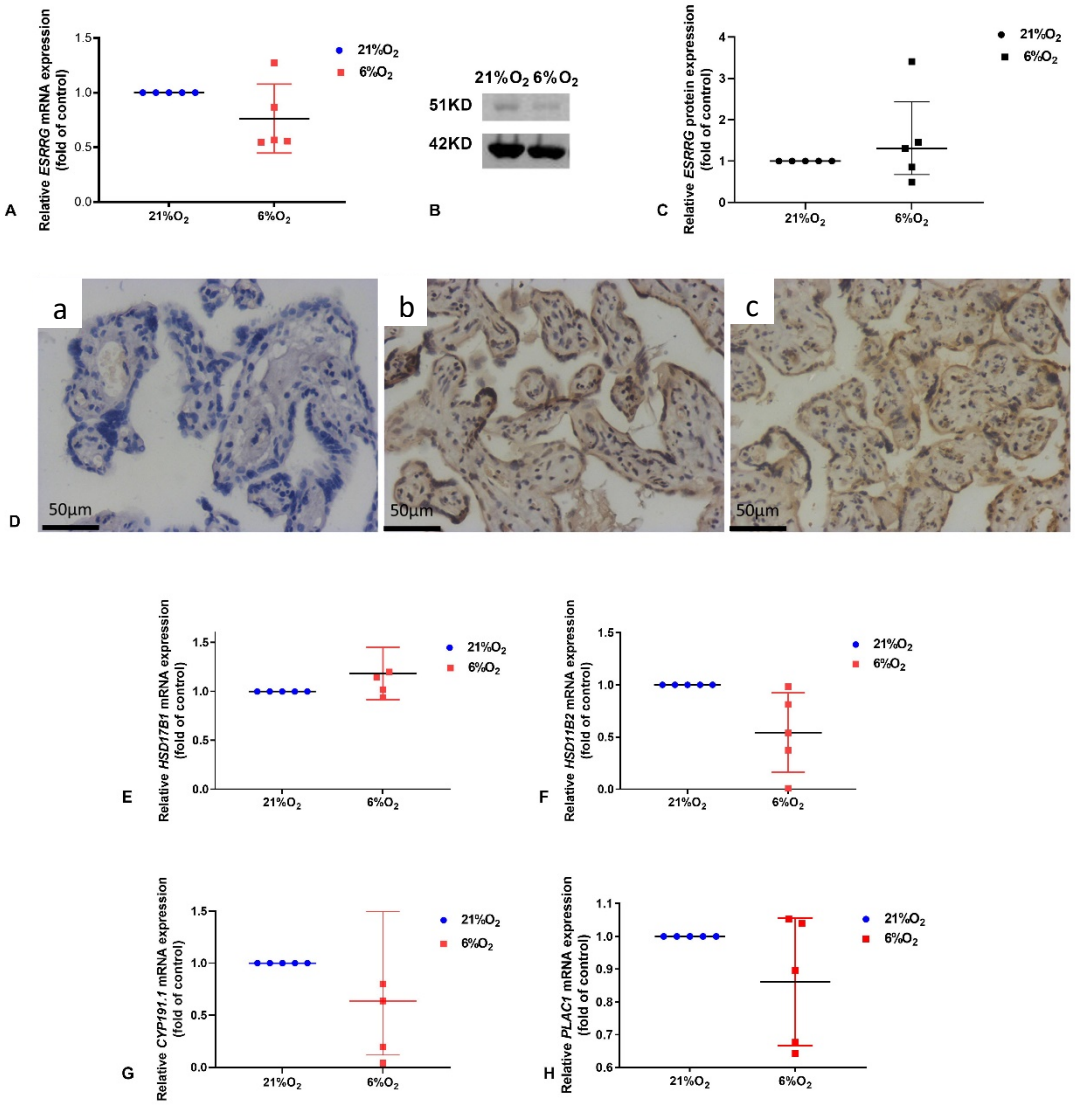
Supplemenatry Figure 3.1. Time course of the mRNA and protein expression of ESRRG in the cultured villous explants between 0-96 hours (day0-day4).

[A] mRNA expression of *ESRRG* . [B, C] Western blotting results; [B] Representative blot showing bands for ESRRG and beta-tubulin; [C] Quantification of the western blot data, normalised to expression of the housekeeping gene, beta-tubulin. [D] mRNA levels of the house-keeping gene, RPLP0, during culture. [E] Quantification of ESRRG protein staining in the villous explants. [F] Representatiive images of ESRRG protein staining, (a), negative control with non-immnue rabbit IgG, (b) fresh placental tissue, (c) explants cultured for 24 hours, (d) explants cultured for 48 hours, (e) explants cultured for 72 hours, and (f) explants cultured for 96 hours. Median+/-IQR. [A] and [C], one sample Wilcoxon test; [D] and [E], Friedman test. *, P<0.05; **P<0.01, n=4.



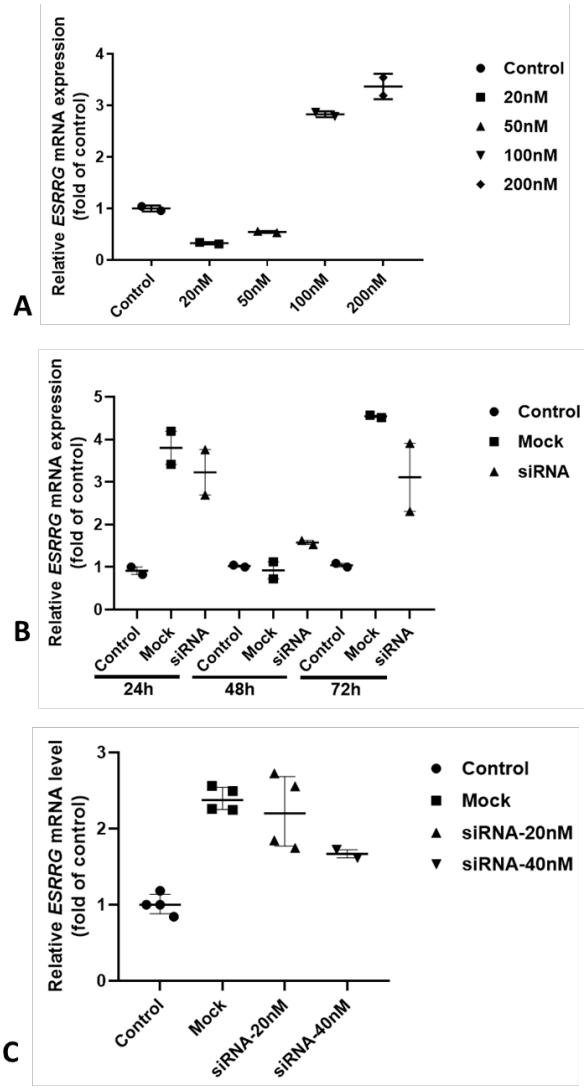
Suppmentary Figure 3.2. Time course of mRNA expression of *ESRRG*'s downstream genes in the cultured villous explants between 0-96 hours (day0 to day4).

Median+/-IQR, one sample Wilcoxon test, n=4. *ESRRG*: oestrogen related receptor gamma; *HSD17B1*: hydroxysteroid 17-beta dehydrogenase 1; *HSD11B2*: hydroxysteroid 11-beta dehydrogenase 2; *CYP191.1*: cytochrome P-450; *PLAC1*: placenta-specific 1.



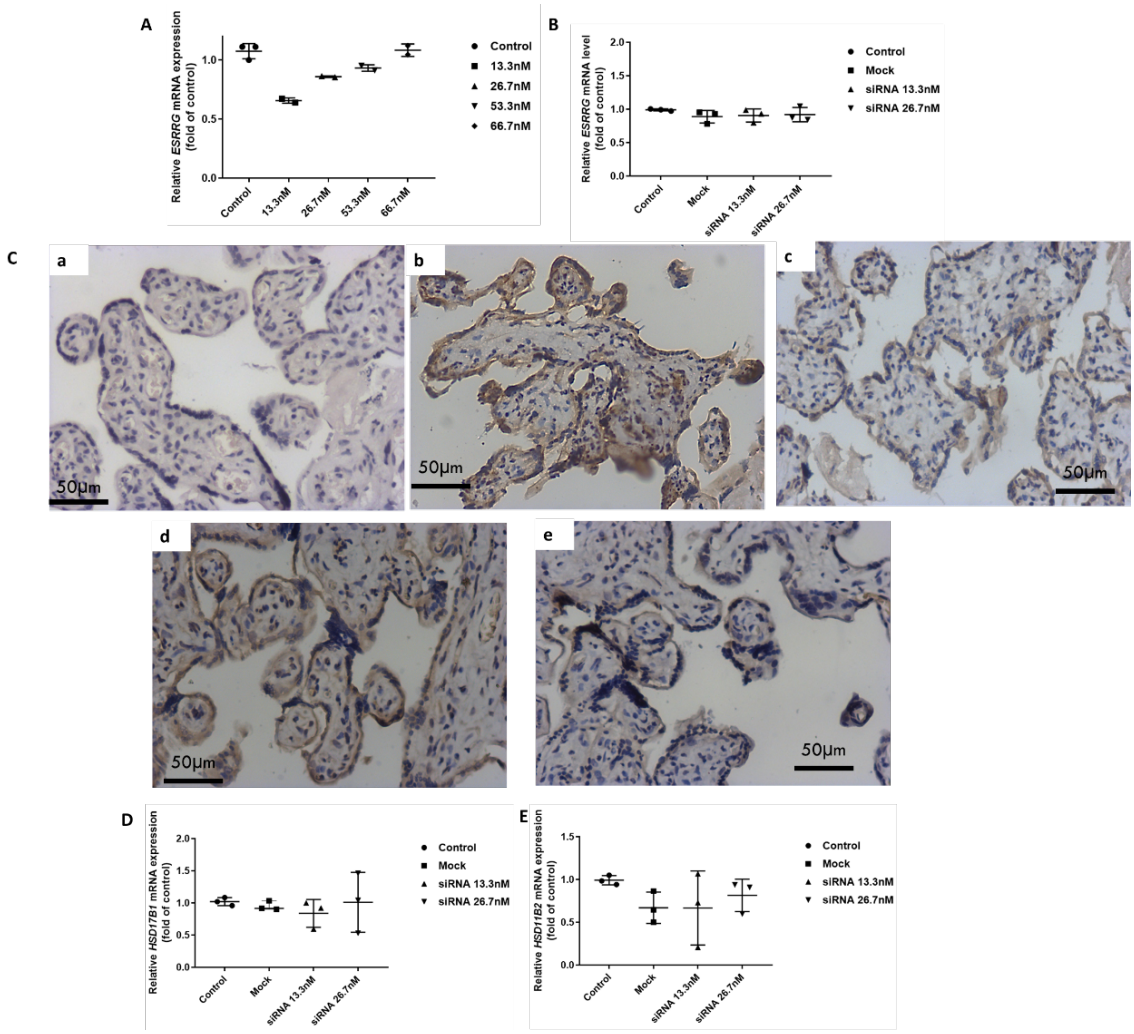
Supplementary Figure 3.3. The mRNA and protein expression of ESRRG and its downstream genes in the villous explants maintained at 21% O_2 compared to 6% O_2.

[A]: mRNA levels of *ESRRG*. [B]: Representative immunoblots for ESRRG (51KD) and beta-actin (42KD). [D]: Quantification of Western blots. [D]: Representative immunostaining images of ESRRG in the villous explants cultured under 21% O_2 [b] or 6% O_2 [c]; [a]: negative control, where non-immune rabbit IgG replaced the primary antibody. [E-H]: mRNA levels of downstream genes. Median+/-IQR; [A-H], one sample Wilcoxon test. n=5.



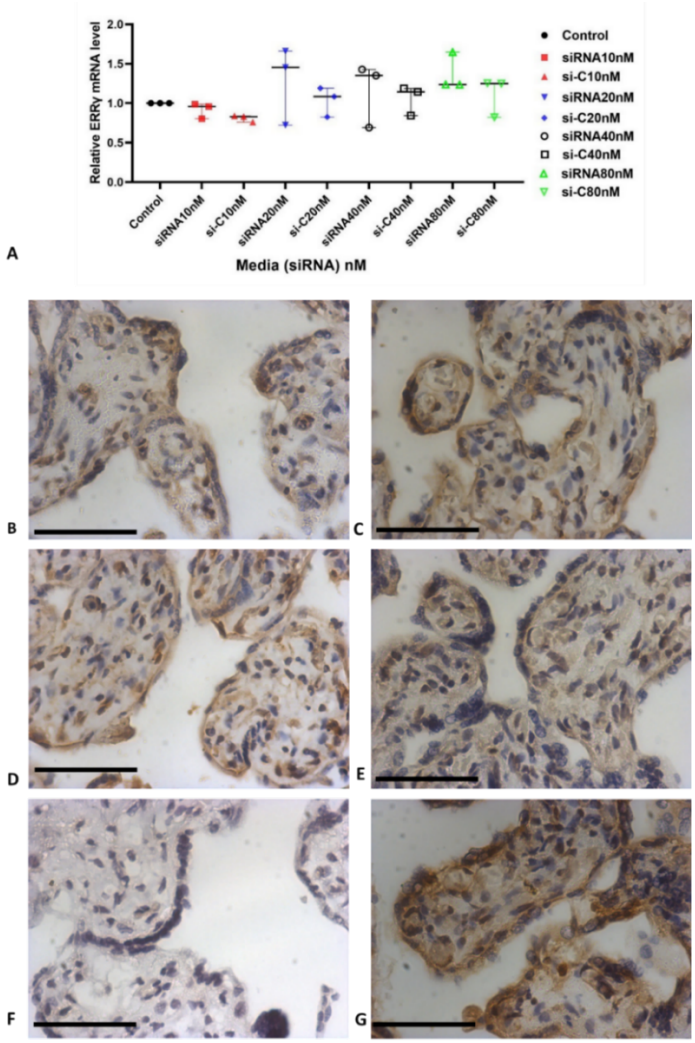
Supplementary Figure 3.4. The transfection of Bewo cells with *ESRRG* siRNA.

[A] Concentration curve and, [B] Time course of *ESRRG* knockdown with 20nM *ESRRG* siRNAs. [C] BeWo cells were transfected with transfection reagent (Mock), *ESRRG* siRNAs (20nM or 40nM) or were untreated (Control). Friedman test, Median+/-IQR.



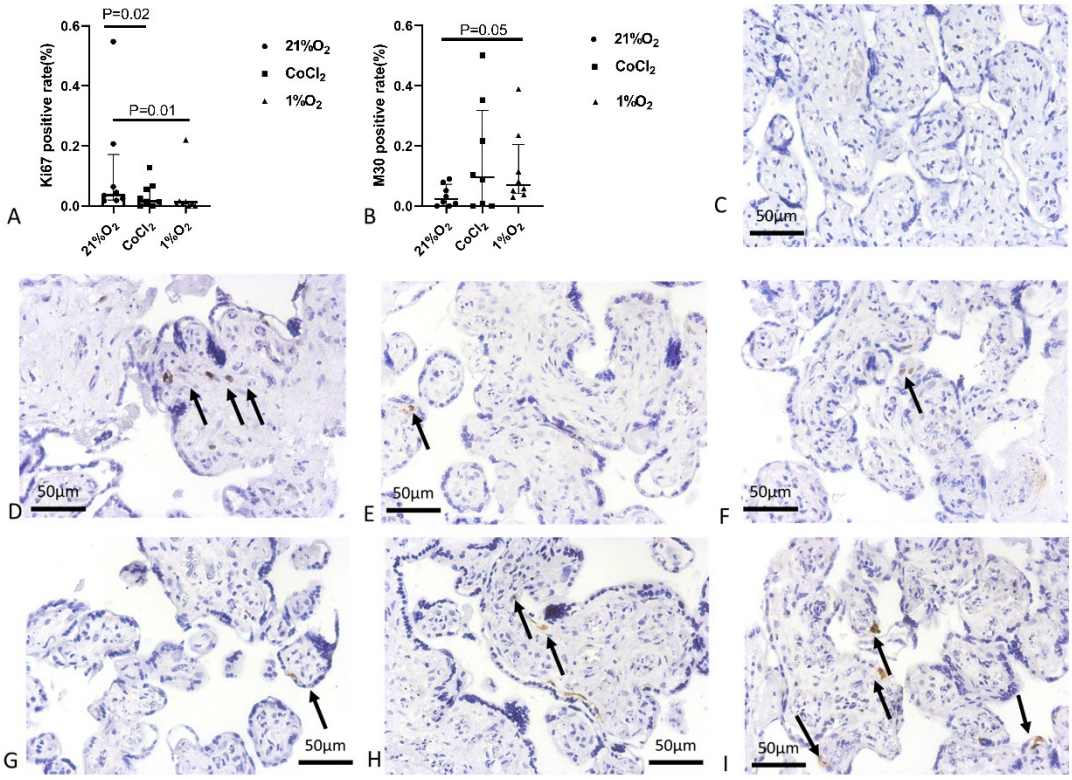
Supplementary Figure 3.5. Term placental villous explants were transfected with different concentrations of *ESRRG* siRNAs and harvested at 48 hours of culture.

[A] Concentration curve. Explants were transfected with 0-66.7nM *ESRRG* siRNAs using transfection reagents; Control, untreated control. [B]: *ESRRG* mRNA expression of untreated explants, or explants treated with transfection reagent (mock) or transfected with 13.3nM or 26.7nM *ESRRG* siRNAs. [C]: Representative immunostaining images of ESRRG; (a), negative control with non-immune rabbit IgG; (b), untreated control; (c), mock transfection; (d), 13.3nM *ESRRG* siRNAs; (e), 26.7nM *ESRRG* siRNAs. [D] and [E]: mRNA expression of *ESRRG*'s downstream genes, *HSD17B1*, and *HSD11B2*. Median+/-IQR; Friedman test. n=3.



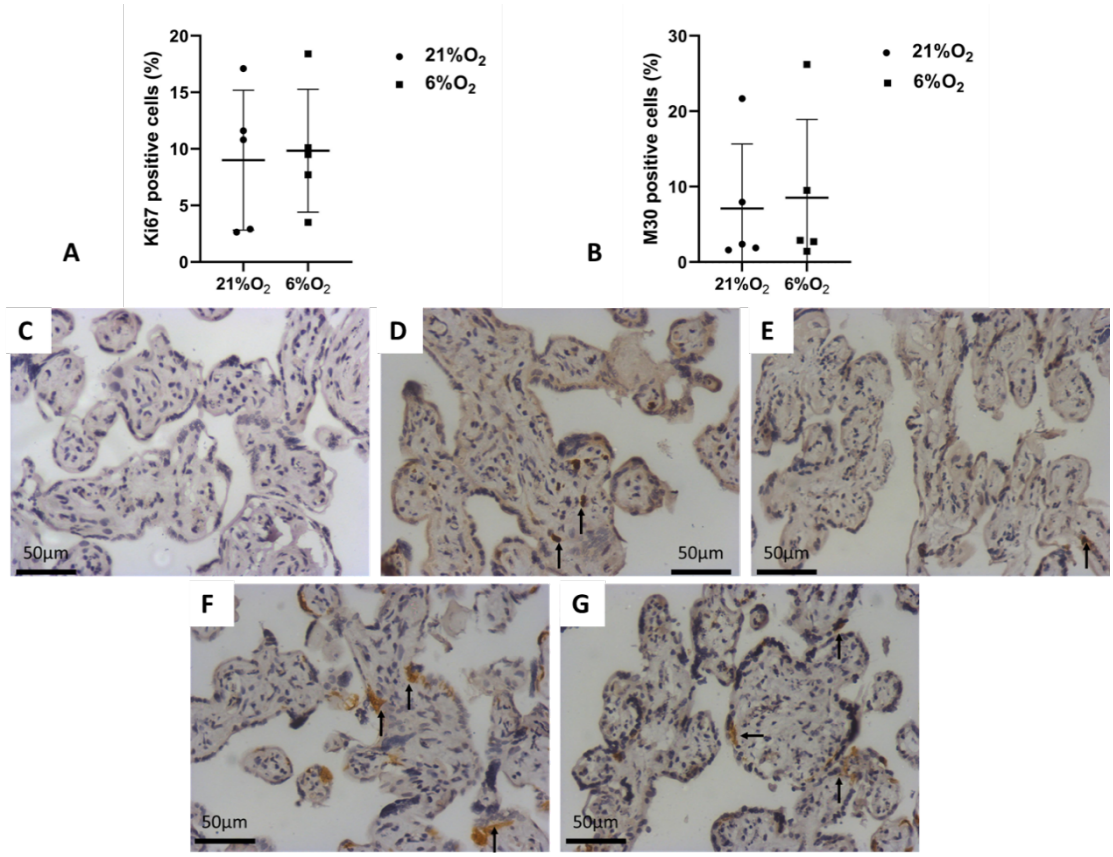
Supplementary Figure 3.6. The mRNA level and protein staining of ESRRG in the term villous explants incubated with 10nM to 80nM *ESRRG* siRNAs, or non-targeting siRNA control (si-C), without transfection reagents.

[A] the mRNA level of *ESRRG* in the transfected cultured villous explants. Control, untreated control; Median+/-IQR; Friedman test. [B-G], Representative photomicrographs of ESRRG protein staining in the transfected villous explants; (B) 40nM *ESRRG* siRNAs; (C) 40nM non-targeting control (si-C); (D) 80nM *ESRRG* siRNAs; (E) 80nM si-C; (F) negative control with non-immune rabbit IgG; (G) untreated control. Explants were harvested at 48 hours of culture after transfection. Scale bar 50µm; nuclei blue; n=3. ESRRG, oestrogen related receptor gamma.



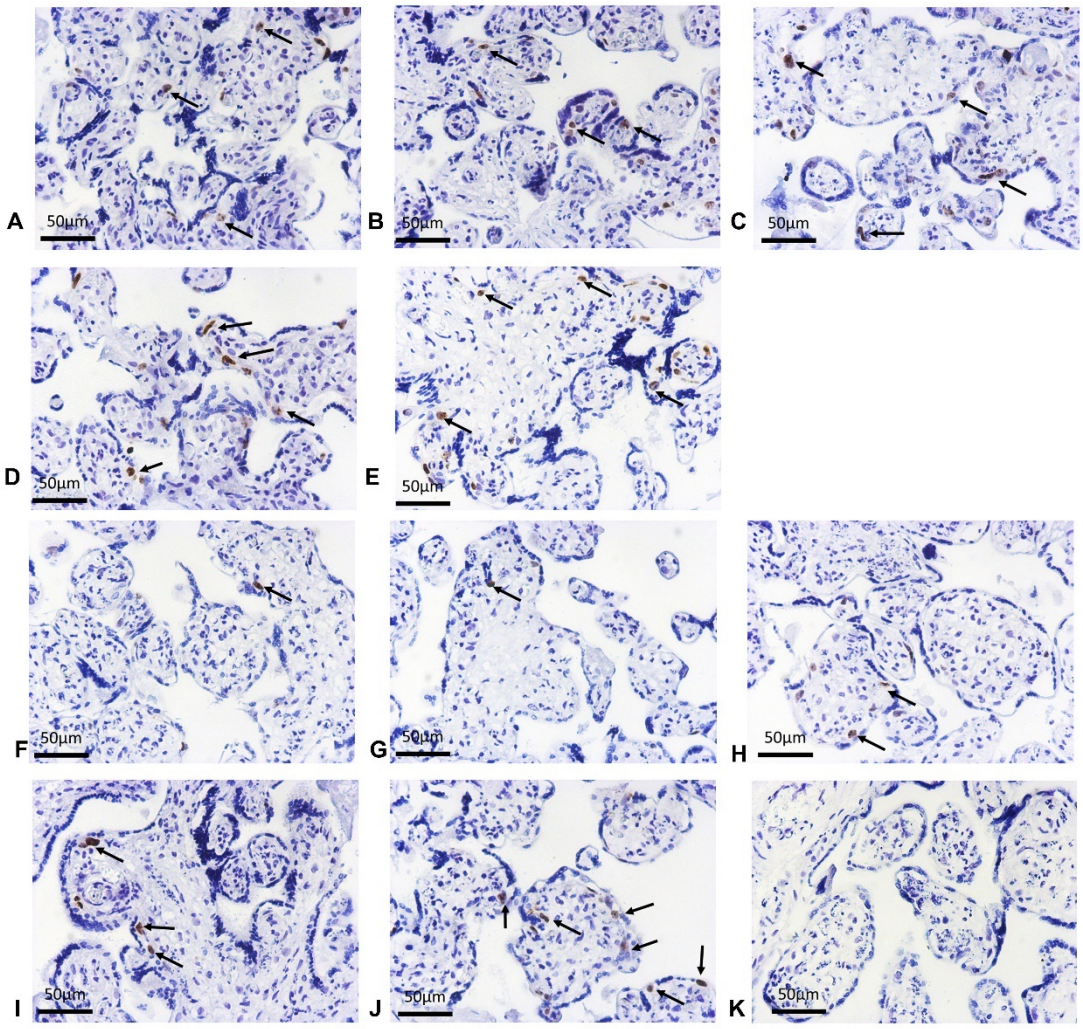
Supplementary Figure 3.7. The Ki-67 and M30 staining results in the cultured villous explants in the different oxygen levels for 96 hours.

[A] and [B] Relative quantification of Ki-67 and M30 staining. [C] Negative control was added non-immune mouse IgG without Ki-67 or M30 primary antibody. [D-F] Representative Ki-67 immunostaining images in the placental explants cultured under 21% O_2 (D), cobalt chloride ($CoCl_2$) treatment (E) or 1% O_2 (F). [G-I] Representative M30 immunostaining images in the explants cultured at 21% O_2 (G), treated with cobalt chloride ($CoCl_2$) (H) or cultured at 1% O_2 (I). Statistical significance was assessed by one-way ANOVA; mean +/- SD. The marker is 50μm.



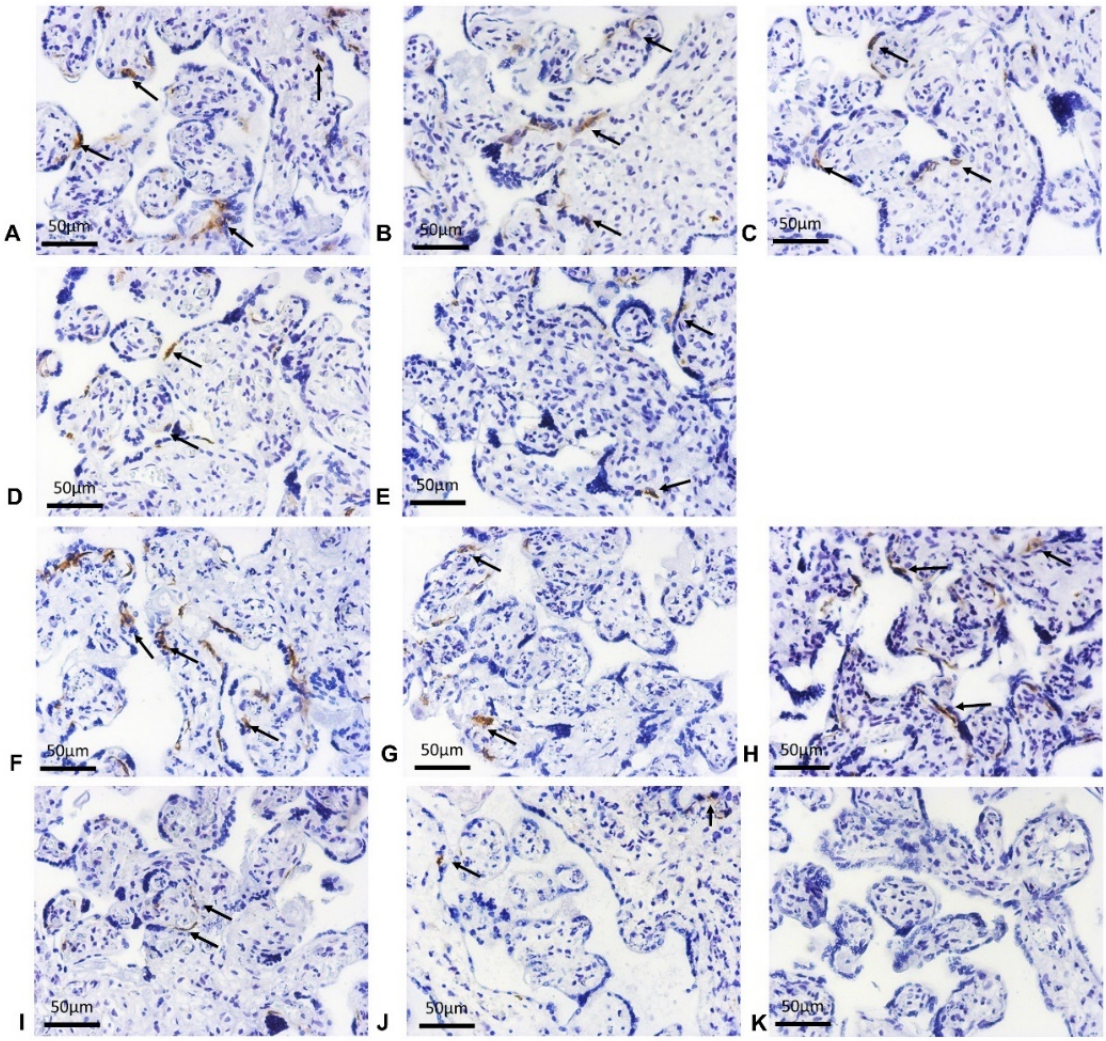
Supplementary Figure 3.8. Immunostaining of Ki-67 and M30 in villous explants cultured under 21%O_2 or 6%O_2 for 96 hours

[A, B] Quantification of Ki-67 and M30 immunostaining. [C-G] Representative images. [C] Negative control with non-immune mouse IgG. [D, E] Ki-67 staining; [F, G] M30 staining. [D] and [F], Represenative immunostaining images in the placental explants cultured at 21% O_2. [E] and [G], Explants cultured at 6% O_2 for 96 hours. Scale bar: 50µM. Wilcoxon matched-pairs signed rank test. n=5.



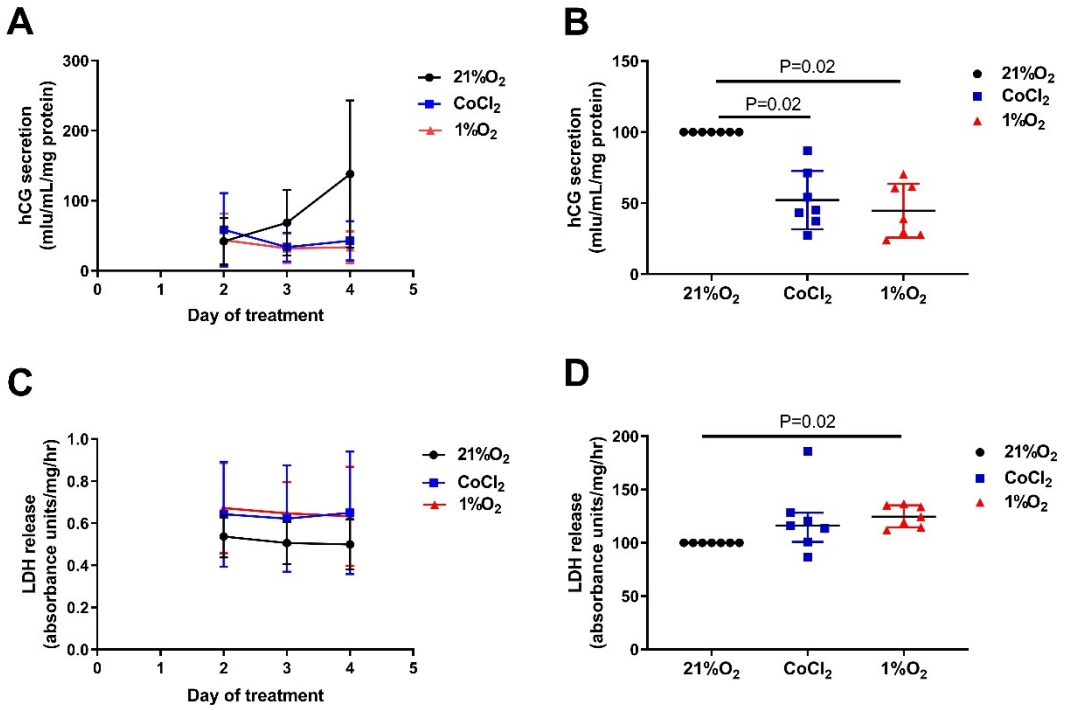
Supplementary Figure 3.9. The Ki-67 staining results in the villous explants with the treatment of GSK5182 or DY131 cultured at 21% O_2 or 1% O_2.

[A-E] Explants cultured at 21% O_2; [F-J] Villous explants cultured in hypoxia (1% O_2). [K] Negative control with the non-immune mouse IgG. [A] and [F], DMSO control; [B] and [G], GSK5182 (20μM); [C] and [H], GSK5182 (50μM); [D] and [I], DY131 (20μM); [E] and [J], DY131 (50μM). Scale bar: 50μM.



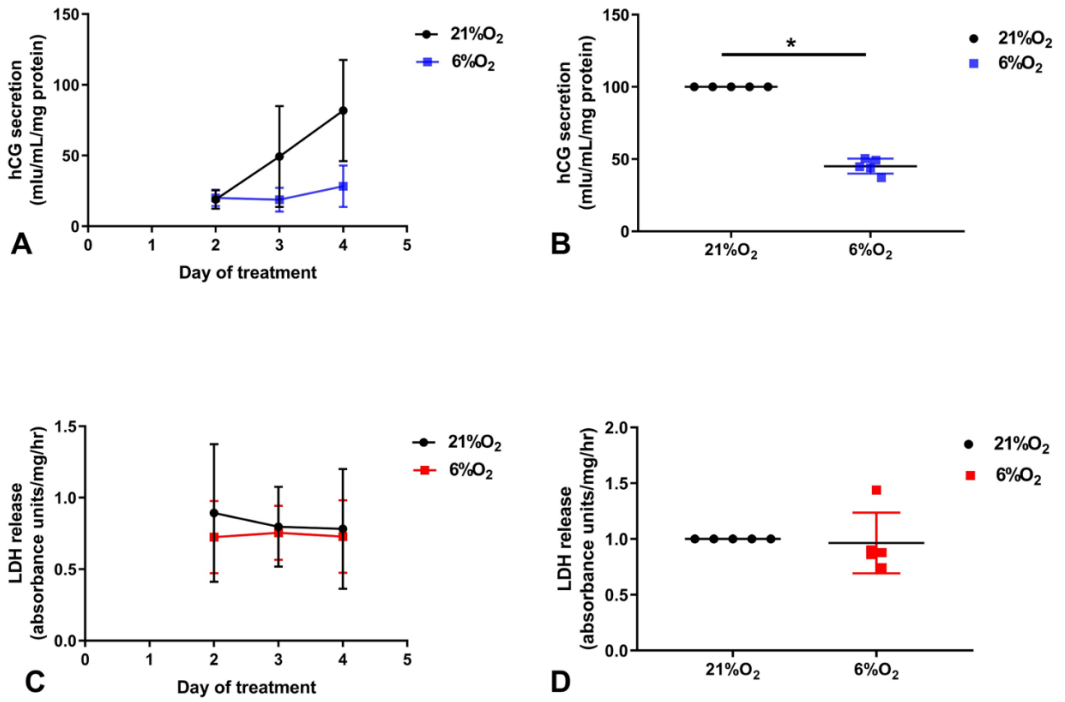
Supplementary Figure 3.10. Representative images of immunostaining for M30 in the villous explants with the treatment of GSK5182 or DY131 under different oxygen concentrations.

[A-E] Explants cultured at 21% O_2; [F-J] Villous explants cultured at 1% O_2. [K] Negative control with the non-immune mouse IgG. [A] and [F], DMSO control; [B] and [G], GSK5182 (20μM); [C] and [H], GSK5182 (50μM); [D] and [I], DY131 (20μM); [E] and [J], DY131 (50μM). Scale bar: 50μM.



Supplementary Figure 3.11. hCG secretion and LDH release from placental explants cultured at different oxygen concentrations.

[A, B]: hCG secretion; [C, D]: LDH release. [A] Time course of hCG secretion from explants cultured under 1% O_2, $CoCl_2$ treatment, or 21% O_2; [B] hCG secretion at day 4 of culture in different oxygen conditions. [C] LDH release over time in the placental explants maintained in 1% O_2, $CoCl_2$ treatment, or 21% O_2; [D] LDH release measured at day 4 from the explants cultured at 1% O_2, $CoCl_2$ treatment, or 21% O_2. Statistical significance was assessed by one sample Wilcoxon test. $CoCl_2$: cobalt chloride; LDH: lactate dehydrogenase; hCG: human chorionic gonadotropin. Mean+/-SD.



Supplementary Figure 3.12. hCG secretion and LDH release in the conditioned culture medium of explants cultured at 21% O_2 or 6% O_2.

[A]: Time course of hCG secretion from explants cultured at 21% O_2 or 6% O_2 during 4 days of culture. [B]: hCG secretion from villous explants cultured at 21% O_2 or 6% O_2 at day 4 of culture. [C]: Time course of LDH release from the villous explants cultured at 6% O_2 or 21% O_2 during 4 days of culture. [D]: LDH levels were measured in the conditioned culture medium at day 4 of culture.

4 CHAPTER 4: Sex Specific Effects of Bisphenol A on the Signalling Pathway of *ESRRG* in the Human Placenta

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ABSTRACT

Bisphenol A (BPA) exposure during pregnancy is associated with low fetal weight, particularly in male fetuses. Expression of oestrogen-related receptor gamma (*ESRRG*), a receptor for BPA in human placenta, is reduced in fetal growth restriction. This study sought to explore whether *ESRRG* signaling mediates BPA-induced placental dysfunction, and determine whether changes in the *ESRRG* signaling pathway are sex-specific. Placental villous explants from 18 normal term pregnancies were cultured with a range of BPA concentrations (1pM-1μM). Baseline BPA concentrations in the placental tissue used for explant culture ranged from 0.04nM-5.1nM (average 2.3nM ±1.9nM; n=6). Expression of *ESRRG* signalling pathway constituents and cell turnover were quantified. BPA (1μM) increased *ESRRG* mRNA expression after 24 hours in both sexes. ESRRG mRNA and protein expression were increased in female placentas treated with 1μM BPA for 24 hours, but were decreased in male placentas treated with 1nM or 1μM for 48 hours. Levels of *HSD17B1* and *PLAC1*, genes downstream of *ESRRG*, were also affected. *HSD17B1* mRNA expression was increased in female placentas by 1μM BPA; however, 1nM BPA reduced *HSD17B1* and *PLAC1* expression in male placentas at 48 hours. BPA treatment did not affect rates of proliferation, apoptosis or syncytiotrophoblast differentiation in cultured villous explants. This study has demonstrated that BPA affects the *ESRRG* signalling pathway in a sex-specific manner in human placentas and provides a possible biological mechanism to explain the differential effects of BPA exposure on male and female fetuses observed in epidemiological studies.

4.1 Introduction

Bisphenol A (BPA), an organic, synthetic compound, has been widely used in plastic products, including food containers, cans, beverages, baby bottles, toys, credit cards, receipts, and medical equipment since the 1950s. BPA concentrations ranging from 0.3 to 17.85 ng/mL (1–78nM) have been observed in adult and fetal plasma, urine, breast milk, and placental tissue (Ikezuki et al., 2002, Welshons et al., 2006, Calafat et al., 2008, Cao et al., 2012, Huo et al., 2015, Lee et al., 2018). Epidemiological and animal studies have shown that maternal BPA exposure is associated with preterm birth, low birth weight, small for gestational age (SGA) infants, fetal growth restriction (FGR), and preeclampsia (Miao et al., 2011, Chou et al., 2011, Cantonwine et al., 2015, Huo et al., 2015, Veiga-Lopez et al., 2015, Muller et al., 2018, Yang et al., 2021). These effects appear to be sex-specific, with male neonates disproportionately affected (Chou et al., 2011). As BPA concentrations in human placenta are four to five times higher than levels in maternal or fetal plasma (Schonfelder et al., 2002), the placenta might play a crucial role in pregnancy disorders related to BPA exposure.

FGR is associated with a range of underlying placental conditions including maternal vascular perfusion, fetal vascular malperfusion and placental inflammation (Spinillo et al., 2019). Maternal vascular malperfusion is the most commonly observed abnormality, particularly in severe early-onset FGR (Spinillo et al., 2019). Our previous studies suggest FGR is also associated with increased placental cell trophoblast apoptosis and disordered proliferation (Heazell et al., 2008, Heazell et al., 2011). However, the mechanism(s) for BPA-related, sex-specific decreases in fetal weight are still unclear and might be related to abnormal placental cell turnover within the placenta, as observed in FGR.

BPA is a weak oestrogen, and although it can bind to oestrogen receptors (ER)- alpha, and ER-beta, progesterone receptor (PR), androgen receptor (AR) and G-protein-coupled receptor 30 (GPCR30) (Takayanagi et al., 2006, Bouskine et al., 2009, Rehan et al., 2015, Liu et al., 2019), BPA is a highly selective agonist for oestrogen related receptor gamma (*ESRRG*) (Brieno-Enriquez et al., 2012, Helies-Toussaint et al., 2014, Tohme et al., 2014, Song et al., 2015), binding with 800 to 1000-fold higher affinity than to other receptors (Takayanagi et al., 2006). Furthermore, a recent study also

suggests that BPA binds to *ESRRG* in a similar manner to natural *ESRRG* ligands (Liu et al., 2019). Both mRNA and protein expression of ESRRG can be induced by short-term BPA exposure in lung cancer cell lines, breast cancer cell lines, adipocytes, hepatocytes, and zebrafish (Helies-Toussaint et al., 2014, Tohme et al., 2014, Song et al., 2015, Zhang et al., 2016, Ryszawy et al., 2020).

ESRRG is highly expressed in the human placenta (Takeda et al., 2009, Poidatz et al., 2012) and levels are reduced in FGR placentas (Poidatz et al., 2012, Poidatz et al., 2015a, Zhu et al., 2018a). Furthermore, *ESRRG* regulates a number of genes in the placenta including: cytochrome P-450 (*CYP191.1*), 17beta-hydroxysteroid dehydrogenase type 1 (*HSD17B1*), 11beta-hydroxysteroid dehydrogenase type 2 (*HSD11B2*) and placenta specific-1 (*PLAC1*) (Shams et al., 1998, McTernan et al., 2001, Homan et al., 2006, Jackman et al., 2012, Luo et al., 2013, Luo et al., 2014, Poidatz et al., 2015a, Muto et al., 2016, Zhu et al., 2018a, Anelli et al., 2019, Zou et al., 2019). Functional studies demonstrate a role for *ESRRG* in regulating proliferation and invasion of the trophoblastic cell line, HTR8/SVneo, and stimulating the differentiation of primary cytotrophoblast from first trimester placentas (Poidatz et al., 2015b, Zhu et al., 2018a, Zou et al., 2019). This evidence demonstrates that *ESRRG* can regulate many aspects of trophoblast function and suggests that dysregulated *ESRRG* signalling may contribute to the pathogenesis of FGR, as well as mediating the effects of BPA on placental dysfunction.

Sex-specific effects of BPA exposure are observed in the offspring's sex differentiation, abnormal neurobehavioral outcomes, skeletal muscle hypertrophy and cardiovascular function in animal models, and the genes mediating these effects include *Esrrg* and *Cyp191.1* (Kubo et al., 2003, Xu et al., 2013, Cao et al., 2013, Kundakovic et al., 2013, Patel et al., 2013, Rosenfeld and Trainor, 2014, Belcher et al., 2015, DeBenedictis et al., 2016, Eckstrum et al., 2016, Ratajczak-Wrona et al., 2019, Jing et al., 2019, Bansal et al., 2020, Raja et al., 2020, Malaise et al., 2021). Even though some previous studies have explored the effects of BPA exposure on the human placenta by using trophoblast cell lines or animal models (Nativelle-Serpentini et al., 2003, Jin and Audus, 2005, Pyo et al., 2007, Benachour and Aris, 2009, Huang and Leung, 2009, Morck et al., 2010, Morice et al., 2011, Tan et al., 2013, De Felice et al., 2015, Rajakumar et al.,

2015, Tait et al., 2015, Sato et al., 2015, Lan et al., 2017, Perez-Albaladejo et al., 2017, Chu et al., 2018, Speidel et al., 2018, Narciso et al., 2019, Xu et al., 2019), only one recent study has investigated the sex-specific effects of BPA on the placenta: Mao et al. used a mouse model of BPA exposure in pregnancy and reported no sex-specific effects (Mao et al., 2020). Therefore, the potential sex-specific effects of BPA on human placenta are yet to be investigated. In addition, only one study has explored the effects of BPA exposure on *ESRRG* expression, using the trophoblast choriocarcinoma cell line JEG-3, which was derived from the placenta of a male fetus (Morice et al., 2011). They observed that 10nM BPA reduced DNA synthesis after 24h and 48h of exposure, which was mediated in part by *ESRRG* signalling. As JEG-3 cells are neoplastic, they are not the most physiologically relevant model for study, thus further work is required to establish the effects of BPA on the *ESRRG* pathway and trophoblast cell turnover in the human placenta.

As *ESRRG* has been implicated as a regulator of signalling pathways underlying placental dysfunction in FGR placentas (Zhu et al., 2018a, Zou et al., 2019), and BPA signals through *ESRRG*, we hypothesised that BPA exposure may initiate or exacerbate placental dysfunction via activation of *ESRRG*. Therefore, this study will explore whether a range doses of BPA (1pM, 1nM, and 1µM) can alter *ESRRG* signaling and cell turnover in the human placental explants, and assess whether the responses are sex-specific.

4.2 Methods

Unless specified, reagents were purchased from Sigma (Sigma, UK).

4.2.1 Placental collection

This study was approved by the Research Ethics Committee (08/H1010/55+5) and written informed consent was collected from all participants. All placental samples were collected from Saint Mary's Hospital, Manchester and were obtained within 30 minutes of delivery. In our study, appropriate for gestational age (AGA) (n=18) was defined as an individualised birth weight ratios (IBR) between the 10th to 90th centile calculated by GROW software (gestation-related optimal weight) (Gestation Network; Birmingham, UK, www.gestation.net)(Gardosi et al., 2018). All participants were non-

smokers, less than 40 years of age and had a BMI of <30 kg/m^2. Women with pregnancy complications such as preeclampsia, chronic hypertension, renal disease, collagen vascular disease, premature rupture of membranes and pregnancies complicated with fetal anomalies or chromosomal abnormalities were excluded from this study.

4.2.2 Placental explant culture

Placental explants were prepared as previously described (Heazell et al., 2008). Briefly, 6 pieces of villous tissue (2cm^3 each) were sampled from normal term placentas from 18 AGA infants (9 males and 9 females), which were further excised as 2mm^3 placental explants and cultured in Netwells (Corning Inc, NY, USA) with 1.5ml culture medium, composed of 1:1 of Dulbecco's modified Eagle medium (DMEM; Gibco, UK)/Ham's F12 (Gibco, UK) supplemented with penicillin (0.6mg/l), streptomycin (100µg/ml), L-glutamine (0.292g/l), and 10% fetal calf serum (Gibco, UK).

The environment-related BPA levels (1nM), and a higher (1µM) and a lower BPA concentration (1pM) were selected for use in this study to explore the effects of a range of relevant BPA concentrations. BPA was dissolved in 50% (v/v) ethanol (with 50% sterile PBS) at a stock concentration of 1 millimolar (mM) and stored at 4°C. The stock solution was further diluted into different concentrations of BPA with culture medium, prior to addition to the villous explants. Fresh BPA dilutions in culture medium were prepared for every experiment, and all products used during culture, such as 12 well plates, netwells, and tips, were BPA-free.

Explants were cultured at 21% O_2 (5% CO_2, 95% air at 37 degrees) for 24 or 48 hours. The villous explants treated with 0.05% (v/v) ethanol were considered as the control group. Conditioned-culture medium and villous tissue was collected at 24 or 48 hours of culture and was processed for ELISA, RNA or protein extraction, or immunohistochemistry analysis.

4.2.3 RNA extraction and RT-PCR

Tissue was homogenised (using SHM1, UK) and total RNA was extracted using a miRNeasy mini kit, (QIAGEN, Germany), following the manufacturer's instructions. 500ng RNA was converted to cDNA using an AffinityScript Multiple Temperature cDNA

according to the manufacturer's instructions. The PCR primer sequences (Eurofins, UK) for *ESRRG* and *RPLP0* (60S acidic ribosomal protein P0) are listed in Table 4.1. *RPLP0* was used as a housekeeping gene and an endogenous control, as it was stably expressed in placental tissues (Zhu et al., 2018a). Powerup SYBR Green Master mix (Thermo Fisher Scientific, USA) was used in the PCR reaction and an Applied Biosystems Step-one system (Thermo Fisher Scientific, USA) was used to run the reaction with an annealing temperature of 60°C, followed by a melt curve step. The fold expression was calculated by the $2^{-\triangle\triangle CT}$ method.

Table 4.1 Primer sequence of ESRRG and its downstream genes

Primer set	Sequence
ESRRG	Forward: 5'-CTG ACG GAC AGC GTC AAC C-3'
	Reverse: 5'-GGC GAG TCA AGT CCG TTC TG-3'
RPLP0	Forward: 5'-TGC ATC AGT ACC CCA TTC TAT CA-3'
	Reverse: 5'-AAG GTG TAA TCC GTC TCC ACA GA-3'
HSD17B1	Forward: 5'-GCC TTC ATG GAG AAG GTG TT-3'
	Reverse: 5'-CGA AAG ACT TGC TTG CTG TG-3'
CYP191.1	Forward: 5'-ACG GAA GGT CCT GTG CTC G-3'
	Reverse: 5'-GTA TCG GGT TCA GCA TTT CCA-3'
PLAC1	Forward: 5'-ATT GGC TGC AGG GAT GAA AG-3'
	Reverse: 5'-TGC ACT GTG ACC ATG AAC CA-3'
HSD11B2	Forward: 5'-GAC CTG ACC AAA CCA GGA GA-3'
	Reverse: 5'-GCC AAA GAA ATT CAC CTC CA-3

ESRRG, oestrogen related receptor gamma; *RPLP0*, 60S acidic ribosomal protein P0; *CYP191.1*, cytochrome P-450; *HSD17B1*, 17 beta-hydroxysteroid dehydrogenase type 1; *HSD11B2*, 11 beta-hydroxysteroid dehydrogenase type 2; *PLAC1*, placenta specific-1.

4.2.4 Analysis of human chorionic gonadotropin (hCG) secretion and lactate dehydrogenase (LDH)

Human chorionic gonadotropin (hCG) is expressed in placental trophoblast and is a marker of cytotrophoblast differentiation. Structurally intact hCG (alpha and beta unit) was quantified in the explant-conditioned culture medium, and in villous explants which were collected at 24h hours or 48 hours and lysed in 0.3M NaOH. The hCG level in conditioned-cultured medium was measured using a hCG ELISA kit (DRG Diagnostics, Marburg, Germany), and a BioRad protein assay (Bio-Rad Laboratories, Hempstead, UK) was used to detect the protein content in the villous explants according to the manufacturer's instructions. The hCG secretion was expressed as mIU/ml/mg explant protein/h.

Lactate dehydrogenase (LDH) is an enzyme which converts lactate to pyruvate in live cells; LDH is released from necrotic cells, thus is a marker of cellular viability (Markert, 1984). LDH release in the explant-conditioned culture medium was quantified as a proxy measure of necrosis in the explants, using a cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. LDH release was expressed as absorbance units/mg explant protein/h.

4.2.5 BPA Content Enzyme-Linked Immunosorbent Assay

Six fresh placentas from 3 male infants and 3 female infants were collected and stored at -80°C prior to sample preparation. Briefly, placental tissue (approximately 1g) was homogenised in 4ml dH_2O at room temperature and 8µl acetic acid was added to acidify the homogenate. After supplying 4ml ethyl acetate, the homogenate was vortexed and centrifuged at 12,000*g* for 3 minutes. The organic phase was collected and dried with nitrogen gas, and the dried residue was dissolved in 20µl ethanol (96%, v/v) with the addition of 500µl sample dilution buffer. Samples were centrifuged at 10,000*g* for 5minutes and then the supernatant was collected for analysis by ELISA (Oestrogen BPA Environmental ELISA Kit, ab175820, Abcam, UK). The ELISA has a sensitivity for BPA measurement ranging from 0.003 ng/ml-1000 ng/ml.

100µl sample or standard were added to a 96 well ELISA plate in duplicate and 100µl HRP-conjugate solution was added to the wells for 2 hours at room temperature. After

washing the wells three times with washing buffer, 200µl TMB substrate was added for 30 minutes at room temperature. 50 µl 2N sulfuric acid was added into the wells to stop the reaction and the absorbance was immediately read at 450nm. The accompanying standard curve was used to calculate the sample BPA concentrations.

4.2.6 Immunostaining

Placental explants were fixed in 4% neutral buffered formalin (NBF) overnight at 4°C and embedded in paraffin wax. 5µm sections were cut and transferred onto the slides pre-coated with poly-L-lysine. After being deparaffinized, the slides were treated for antigen retrieval by microwave boiling (800W, 10 minutes), and then incubated with 3% (v/v) hydrogen peroxide for 10 minutes. Slides were incubated with the non-immune block (10% goat serum and 2% human serum in 0.1% TBST (TBS-Tween-20) for 30 minutes at room temperature and then incubated with a polyclonal antibody against ESRRG (Abcam 215947, 10µg/ml), a monoclonal antibody against Ki67 (Dako, 0.17µg/ml), or a monoclonal antibody against M30 (Roche, 0.13µg/ml) overnight at 4°C. The negative control was an isotype-specific non-immune rabbit or mouse IgG used at the same concentration as the primary antibodies. Immunostaining for Ki67 and M30 was carried out as markers of cycling cells, and hence were proxy markers of proliferation and apoptosis respectively (Heazell et al., 2008, Renshall et al., 2021). The secondary antibody (biotin-conjugated goat anti-mouse or anti-rabbit antibodies, Dako-Cytomation, UK, 3.85 µg/ml) was applied and incubated for 30 minutes at room temperature, followed by incubation with avidin-peroxidase (5µg/ml) for 30 minutes. Chromogenic substrate diaminobenzidine (DAB; Sigma-Aldrich, UK) was applied to the sections for between 2-10 minutes; colour development was monitored under the microscope. All slides were counterstained with Harris's hematoxylin (Sigma-Aldrich, UK) for 5 minutes, and then differentiated with acid alcohol for 2 seconds. All villous explants for comparison were stained in the same batch and all negative control samples had no immunoactivity.

4.2.7 Statistical analysis

All data are presented as the mean ± standard deviation (SD) (normally distributed) or median ± interquartile range (IQR) (non-normally distributed); the Shapiro-Wilk test was used to determine whether the data were normally distributed. Statistical analysis

was undertaken with GraphPad Prism version 7.0 (GraphPad Software, USA). Data were assessed using one sample Wilcoxon test or Kruskal-Wallis, followed by a Friedman multiple comparison test for non-parametric data. QuPath version 0.2.3 (developed by University of Edinburgh) was used to analyze the IHC staining results (Bankhead et al., 2017). The detection of positively stained cells was used to quantify the extent of Ki67 and M30 staining, which was expressed as a percentage the total number of cells. For ESRRG protein staining, the percentage of DAB-positive area to total tissue area was calculated. A P value <0.05 was considered as statistically significant.

4.3 Results

4.3.1 Demographic characteristics

The demographic characteristics of participants are listed in Table 4.2. The age, BMI, gestational age, and ethnicity of pregnant women were comparable between male and female fetuses' group, as well as the fetal weight and Apgar score.

4.3.2 BPA alters *ESRRG* expression in a sex-specific manner in term villous explants

Baseline BPA concentrations in the placental tissue used for explant culture ranged from 0.04nM-5.1nM (average 2.3nM ±1.9nM; n=6). The BPA concentrations were similar in placentas from male and female infants (male mean 2.2nM ±2.0nM vs. 2.4nM±1.8nM in females, Figure 4.1). The effects of in-vitro BPA treatment (1pM-1μM above baseline), on the mRNA and protein expression of ESRRG in term placental villous explants cultured for 24 or 48 hours, was then examined (Figure 4.2). mRNA expression of *ESRRG* was significantly increased following exposure to 1μM BPA for 24h, but there was no change in expression after treatment with 1pM or 1nM BPA (Figure 4.2A). After 48 hours, BPA treatment did not significantly alter mRNA or protein expression of ESRRG at any concentration (Figure 4.2A and 4.2B). There was no change in the localisation of ESRRG following BPA treatment (Figure 4.2D-K), which was predominantly found in the syncytiotrophoblast layer and endothelium of the villous.

When data from male and female placentas were separated by sex, BPA treatment led to a sex-specific alteration of *ESRRG* expression; 1μM BPA significantly increased the mRNA level of *ESRRG* in female placentas after 24 hours (Figure 4.3A, $P<0.01$), which was consistent with the protein expression data (Figure 4.3B, $P<0.05$). We also observed that ESRRG protein staining is mainly localised in the cytoplasm of syncytiotrophoblast, and whilst overall levels increased, localisation was unaffected by BPA treatment (Figure 4.3D-2K). In the untreated placental explants, the mRNA levels of *ESRRG* and its downstream genes were comparable between male infants and female infants (Figure 4.4).

After 48 hours, the mRNA level of *ESRRG* was significantly decreased in placental explants from male infants following treatment with 1nM or 1µM BPA (Figure 4.5A). Quantification of ESRRG protein expression confirmed the reduction of ESRRG with 1nM BPA treatment in male villous explants (Figure 4.5B, $P<0.05$). The localisation of ESRRG protein in male placentas (Figure 4.5D-4.5G) did not appear to differ from female placentas (Figure 4.5H-4.5K).

Table 4.2 Demographics of pregnant women and fetuses.

Characteristics	Male group (n=9)	Female group (n=9)	P value
Birth weight (g)	3410.0±625.0	3436.0±391.0	NS
Gestational age at delivery (weeks)	39.2	38.9	NS
Maternal age (years)	32.8±2.8	29.9±4.8	NS
Parity (primary/multiple)	2/7	1/8	
BMI (kg/m^2)	26.4±3.3	24.7±5.0	NS
Ethnicity			
White British	6 (66.7%)	4(44.4%)	NS
Indian	0	1(11.1%)	
Pakistani	1(11.1%)	2(22.2%)	
Black	1(11.1%)	1(11.1%)	
Other	1(11.1%)	1(11.1%)	
Apgar score (at 5 minutes)	10	10	NS

Data were presented as mean ± standard deviation (SD) or median ± interquartile range (IQR). NS, no significance.

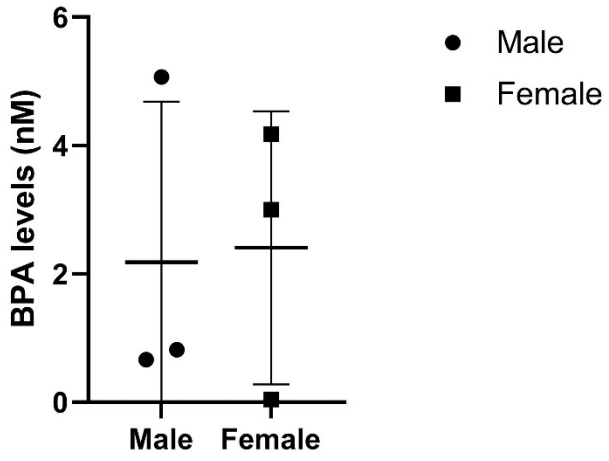

Figure 4.1 BPA concentrations in the fresh placental tissues.

Mean ± SD. Unpaired t test.

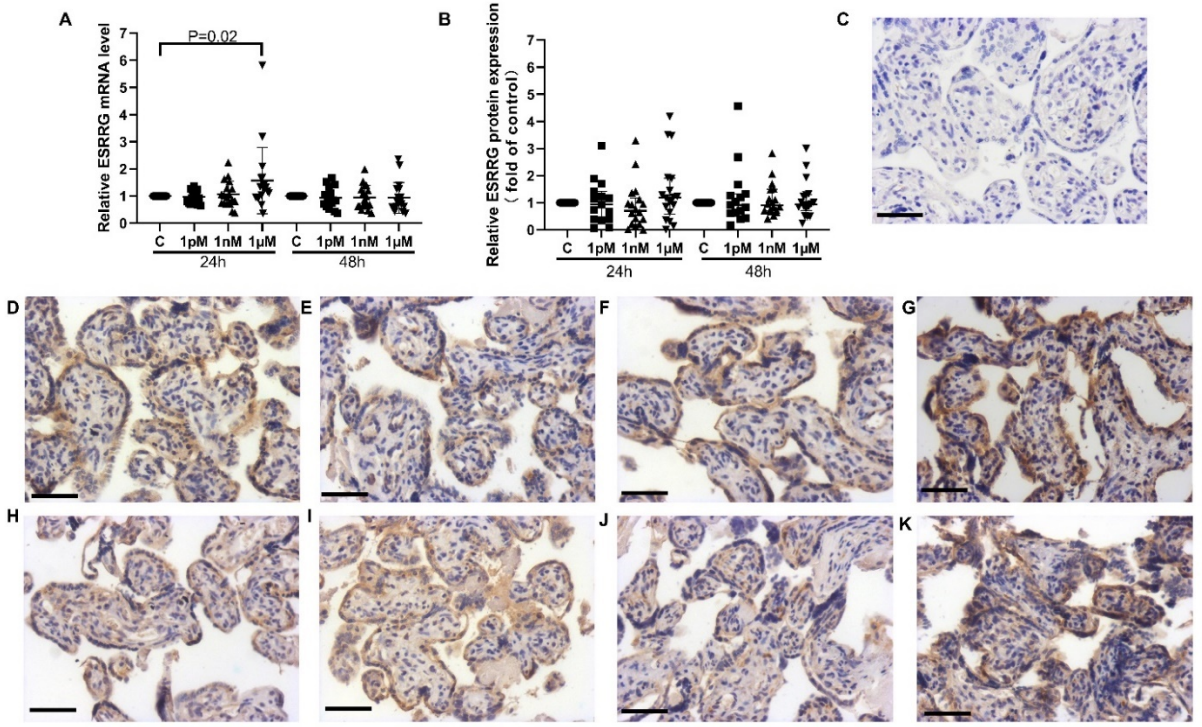

Figure 4.2 Effects of BPA exposure on expression of ESRRG in term villous explants.

Figure 4.2A, mRNA expression of *ESRRG*. Figure 4.2B, Quantification of ESRRG immunostaining. Figure 4.2C-J, Representative images of immunostained cultured villous explants. Figure 4.2C, Negative control. Figure 4.2D-G, 24 hours of culture; Figure H-K, 48 hours of culture. Figure 4.2D and 4.2H, Controls (0.05% (v/v) ethanol); 4.2E and 4.2I, 1pM BPA; 4.2F and 4.2J, 1nM BPA; 4.2G and 4.2K, 1µM BPA. One sample Wilcoxon test, median+/-IQR. n=18. Bar = 50 µm.

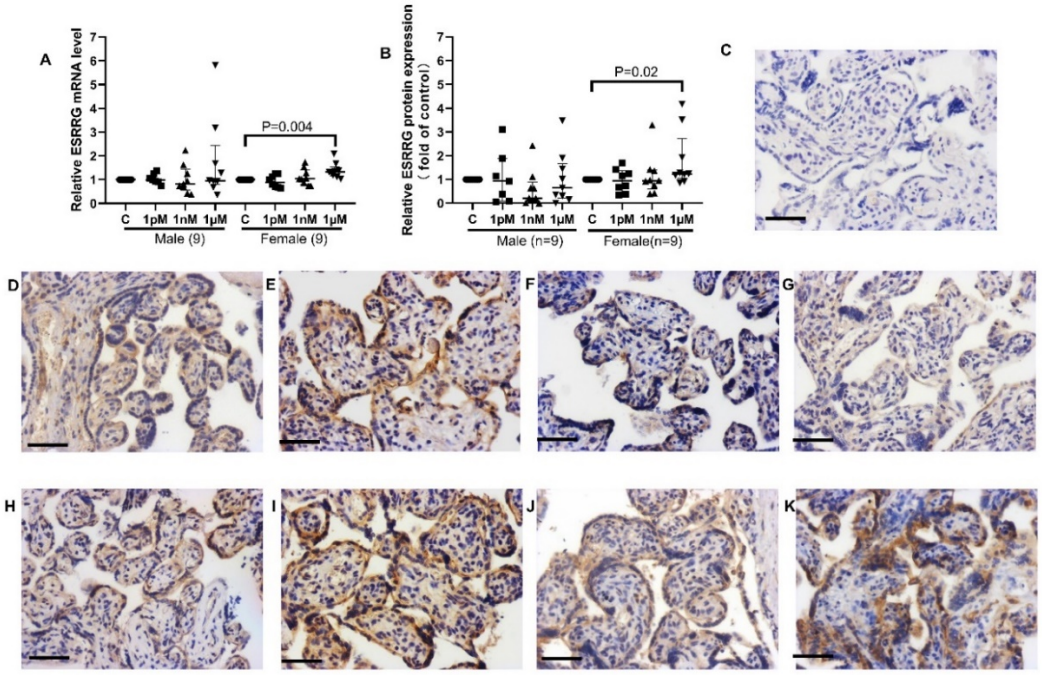

Figure 4.3 mRNA and protein expression of ESRRG in term villous explants from male and female fetuses after BPA exposure for 24 hours.

Figure 4.3A, mRNA expression of *ESRRG*. Figure 4.3B, Quantification of ESRRG immunostaining. Figure 4.3C, Negative control. Representative images of immunostained villous explants from male infants (4.3D-G), and female fetuses (4.3H-K). Figure 4.3D and 4.3H, Controls (0.05% (v/v) ethanol). Figure 4.3E and 4.3I, 1pM BPA. Figure 4.3F and 4.3J, 1nM BPA. Figure 4.3G and 4.3K, 1μM BPA. One sample Wilcoxon test, median+/- IQR. Bar = 50 μm.

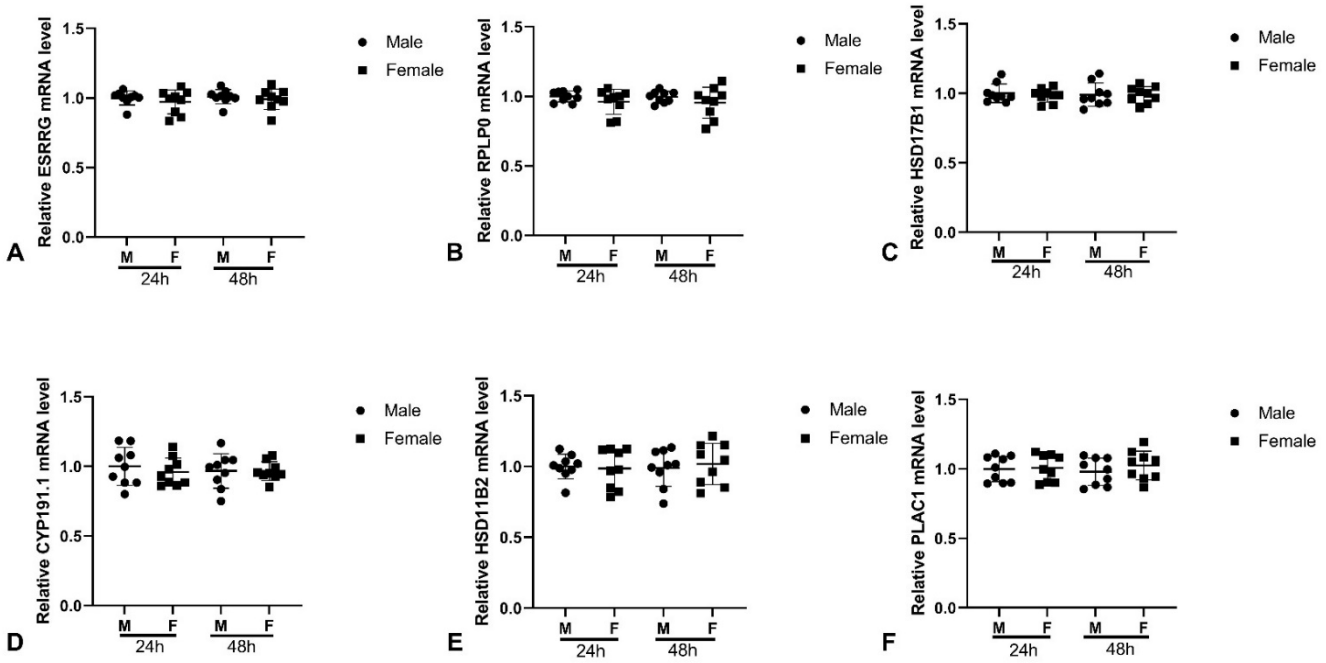

Figure 4.4 mRNA expression of ESRRG, RPLP0, and downstream genes in the untreated villous explants from male or female fetuses for 24 hours or 48 hours of culture.

The CT value of male group (24h) was used to normalise other three groups. Mean ± SD. *CYP191.1*, cytochrome P-450; *HSD17B1*, 17beta-hydroxysteroid dehydrogenase type 1; *HSD11B2*, 11beta-hydroxysteroid dehydrogenase type 2; *PLAC1*, placenta specific-1.

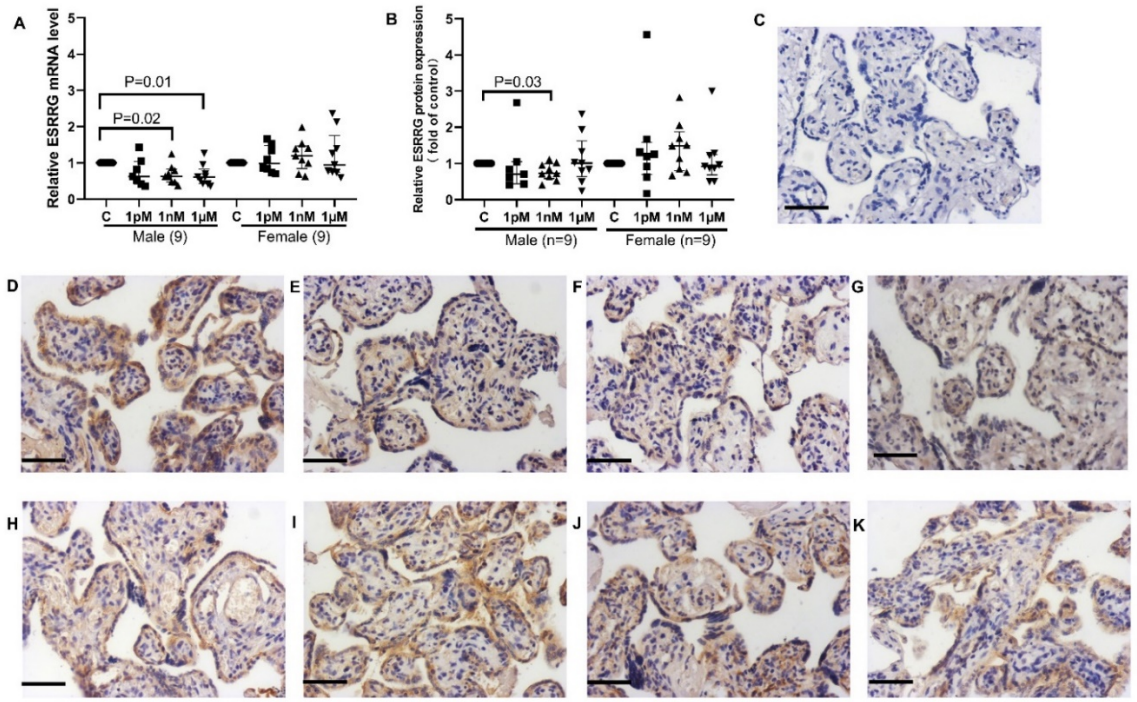

Figure 4.5 mRNA and protein expression of ESRRG in male or female villous explants treated with BPA for 48 hours.

Figure 4.5A, mRNA expression of *ESRRG*. Figure 4.5B, Quantification of ESRRG immunostaining. Figure 4.5C, Negative control. Representative images of immunostained placental explants from male fetuses (4.5D-G), and female infants (4.5H-K). Figure 4.5D and 4.5H, Control (0.05% (v/v) ethanol). Figure 4.5E and 4.5I, 1pM BPA. Figure 4.5F and 4.5J, 1nM BPA. Figure 4.5G and 4.5K, 1µM BPA. One sample Wilcoxon test, median+/- IQR. Bar = 50 µm.

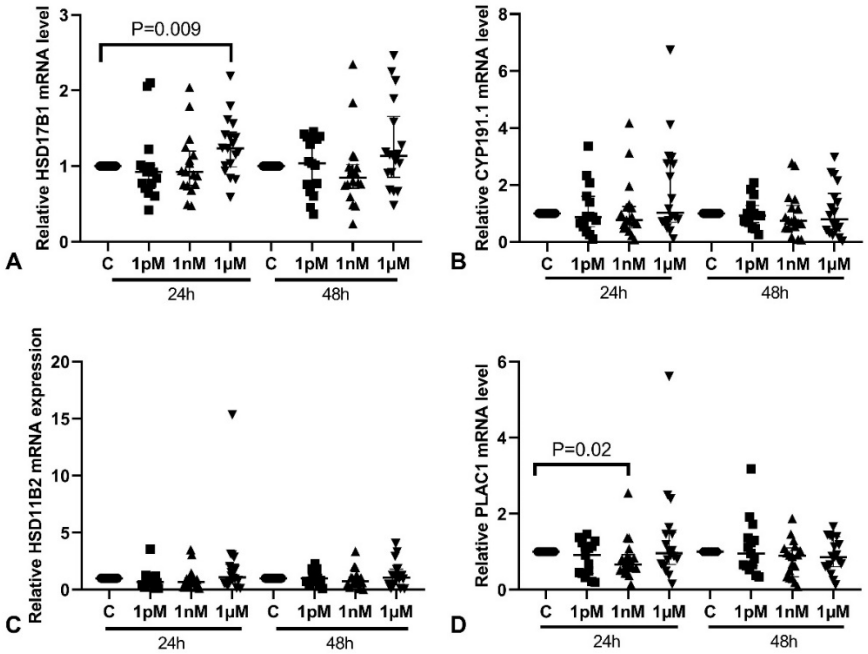

Figure 4.6 Effects of BPA on the mRNA levels of downstream genes of ESRRG.

Figure 4.6A, *HSD17B1*; 4.6B, *CYP191.1*; 4.6C, *HSD11B2*; 4.6D, *PLAC1*. One sample Wilcoxon test; n=18. Median+/-IQR. *CYP191.1*, cytochrome P-450; *HSD17B1*, 17 beta-hydroxysteroid dehydrogenase type 1; *HSD11B2*, 11 beta-hydroxysteroid dehydrogenase type 2; *PLAC1*, placenta specific-1.

4.3.3 BPA alters mRNA expression of genes downstream of *ESRRG* in a sex-specific manner

To assess the effects of BPA on *ESRRG* signalling, we measured the mRNA expression of several genes downstream of *ESRRG*, including *HSD17B1*, *CYP191.1*, *HSD11B2*, and *PLAC1*. In the villous explants from both male and female placentas, the mRNA level of *HSD17B1* was significantly increased after treatment with 1μM BPA for 24h (Figure 4.6A, $P<0.01$). mRNA expression of *CYP191.1* and *HSD11B2* was unchanged at 24h following BPA treatment (Figure 4.6B and 4.6C). The mRNA level of *PLAC1* was significantly reduced after treatment with 1nM BPA for 24 hours, compared to control explants ($P<0.05$, Figure 4.6D). No changes in mRNA expression of these four genes were observed after 48 hours of BPA treatment.

Sex-specific effects of BPA on the mRNA levels of these four genes were also observed. In placentas from female fetuses, compared to the control group, the mRNA expression of *HSD17B1* increased following treatment with 1μM BPA for 24 hours ($P=0.05$, Figure 4.7A) or 48 hours of culture ($P=0.05$, Figure 4.7B). Meanwhile, the mRNA expression of four downstream genes was significantly decreased in the villous explants from male fetuses following treatment with 1nM BPA for 48 hours (Figure 4.7B, 4.7D, 4.7F, and 4.7H).

4.3.4 BPA effects on trophoblast differentiation and necrosis

BPA treatment did not change the level of hCG secreted into the culture medium at any concentration tested at 24h or 48 hours (Figure 4.8A). LDH release was modestly but significantly increased after treatment with 1pM BPA for 24h, but levels were comparable in the villous explants cultured for 48 hours (Figure 4.8D, $P<0.05$). There was no evidence of sexually dimorphic responses in the hCG secretion or LDH release in response to BPA exposure (Figure 4.8B, 4.8C, 4.8E, and 4.8F).

4.3.5 BPA effects on Ki67 and M30 expression

Compared to the control group, the percentage of cells in cycle (Ki67-positive cells) and apoptotic cells (M30-positive cells) was not affected by any concentration of BPA at 24 hours and 48 hours in the cultured explants (Figure 4.9 and Figure 4.10), and no

sexually dimorphic responses were observed in these explants with BPA exposure (Figure 4.11, Figure 4.12, Figure 4.13 and Figure 4.14).

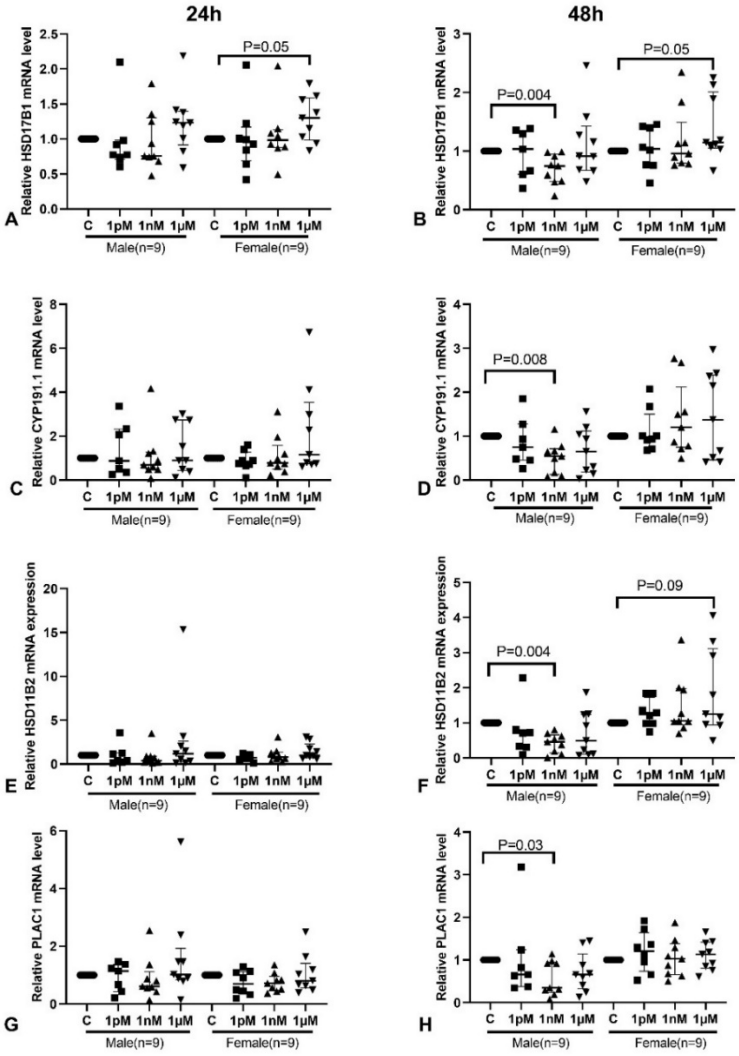

Figure 4.7 The sex-specific effects of BPA on the mRNA levels of downstream genes of ESRRG.

Figure 4.7A (*HSD17B1*, 24h) and 4.7B (*HSD17B1*, 48h); Figure 4.7C (*CYP191.1*, 24h) and 4.7D (*CYP191.1*, 48h); Figure 4.7E (*HSD11B2*, 24h) and 4.7F (*HSD11B2*, 48h). Figure 4.7G (*PLAC1*, 24h) and 4.7H (*PLAC1*, 48h), mRNA levels. One sample Wilcoxon test, median+/-IQR. *CYP191.1*, cytochrome P-450; *HSD17B1*, 17 beta-hydroxysteroid dehydrogenase type 1; *HSD11B2*, 11 beta-hydroxysteroid dehydrogenase type 2; *PLAC1*, placenta specific-1.

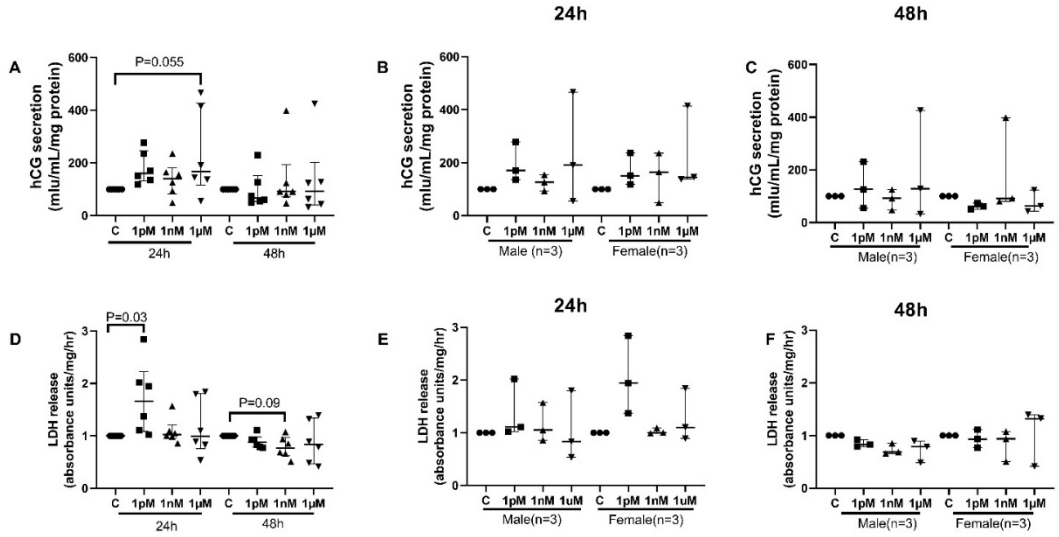

Figure 4.8 hCG and LDH levels in explant culture medium following BPA exposure.

Figure 4.8A hCG, and 4.8D LDH levels in the culture medium from all villous explants after BPA treatment for 24 or 48 hours (n=6). Figure 4.8B, hCG levels in culture medium from explants from male and female for 24 hours of culture, and Figure 4.8C, for 48 hours of culture. Figure 4.8E LDH levels in culture medium from explants from male and female for 24 hours of culture, and Figure 4.8F, for 48 hours of culture. n=6. Median+/-IQR, one sample Wilcoxon test.

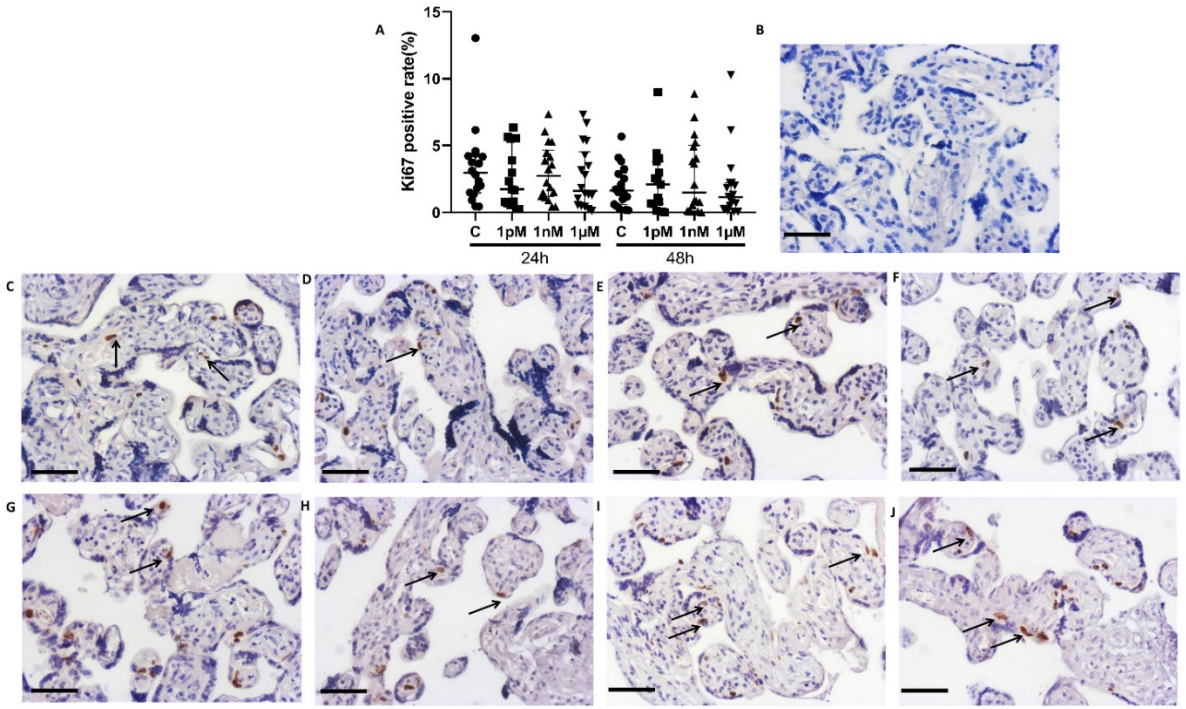

Figure 4.9 Effects of BPA on the percentage of cells in cycle in villous explants cultured for 24 hours or 48 hours.

Figure 4.9A, Quantification of Ki67 staining. Figure 4.9B, Negative control. Representative images of Ki67 staining in the placental explants cultured for 24 hours (Figure 4.9C-F) or 48 hours (Figure 4.9G-J). Figure 4.9C and 4.9G, Control group (0.05% ethanol); 4.9D and 4.9H, 1pM BPA; 4.9E and 4.9I, 1nM BPA; 4.9F and 4.9J, 1μM BPA. Black arrow, Ki67 positive cells. Kruskal-Wallis test, median+/-IQR. n=18. Bar = 50 μm.

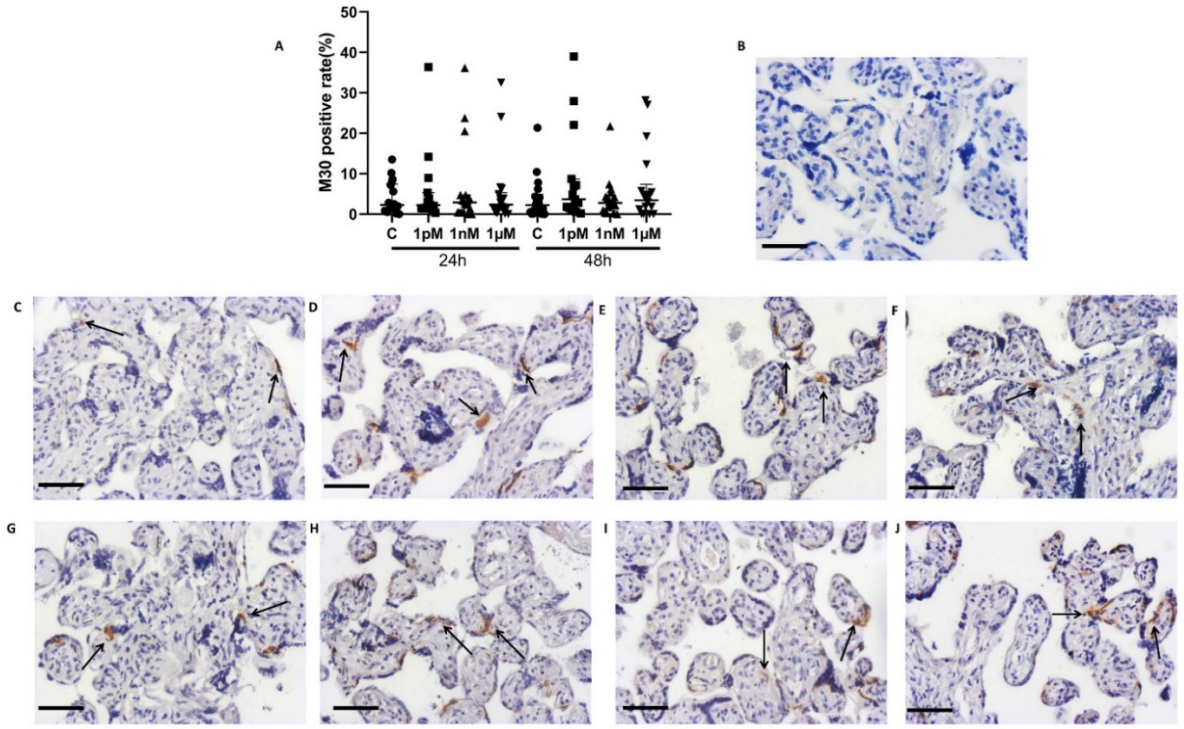

Figure 4.10 Effects of BPA on the percentage of apoptotic cells in villous explants cultured for 24 hours or 48 hours.

Figure 4.10A, Quantification of M30 staining. Figure 4.10B, Negative control. Representative images of M30 staining after 24 hours (Figure 4.10C-F) or 48 hours (Figure 4.10G-J) of BPA exposure. Figure 4.10C and 4.10G, Control (0.05% (v/v) ethanol); 4.10D and 4.10H, 1pM BPA; 4.10E and 4.10I, 1nM BPA; 4.10F and 4.10J, 1µM BPA. Black arrow, M30 positive cells. Kruskal-Wallis test, median+/-IQR. n=18. Bar = 50 µm.

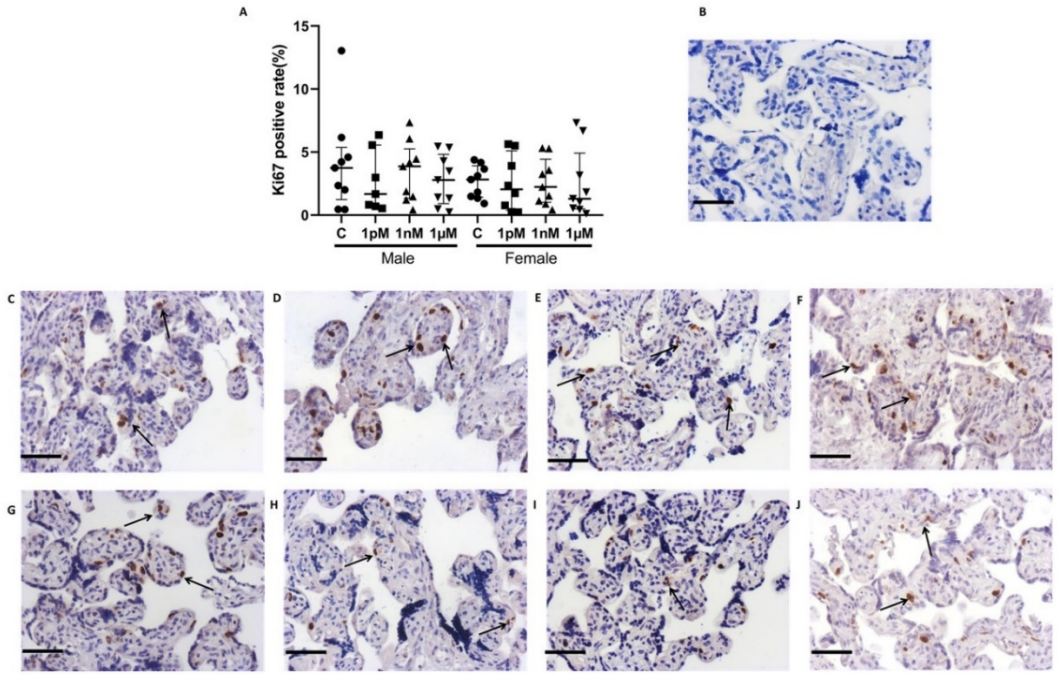

Figure 4.11 The effect of BPA on the percent of Ki67 positive cells in male and female placental explants for 24 hours of culture.

Figure 4.11A, Quantification of Ki67 positive cells. Figure 4.11B, Negative control. Representative images of immunostained placental explants from male fetuses (Figure 4.11C-F), and female infants (Figure 4.11G-J). Figure 4.11C and Figure 4.11G, Control (0.05% (v/v) ethanol). Figure 4.11D and Figure 4.11H, 1pM BPA. Figure 4.11E and I, 1nM BPA. Figure 4.11F and J, 1μM BPA. Black arrow, Ki67 positive cells. Kruskal-Wallis test, median+/-IQR. n=18. Bar = 50 μm.

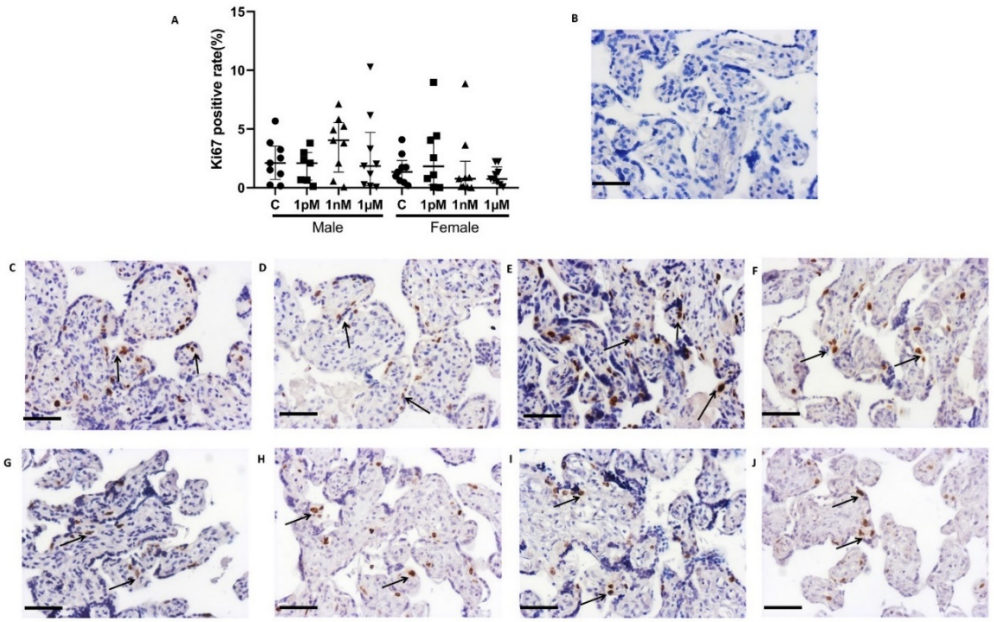

Figure 4.12 Effects of BPA on the percent of Ki67 positive cells in the placental villous explants from male and female fetuses for 48 hours of culture.

Quantification of Ki67 positive cells was listed in Figure 4.12A. [B], Negative control. Representative images of immunostained placental explants from male fetuses (C-F), and female infants (G-J). Figure 4.12C and Figure 4.12G, Control (0.05% (v/v) ethanol). Figure 4.12D and Figure 4.12H, 1pM BPA. Figure 4.12E and Figure 4.12I, 1nM BPA. Figure 4.12F and Figure 4.12J, 1μM BPA. Black arrow, Ki67 positive cells. Kruskal-Wallis test, median+/-IQR. n=18. Bar = 50 μm.

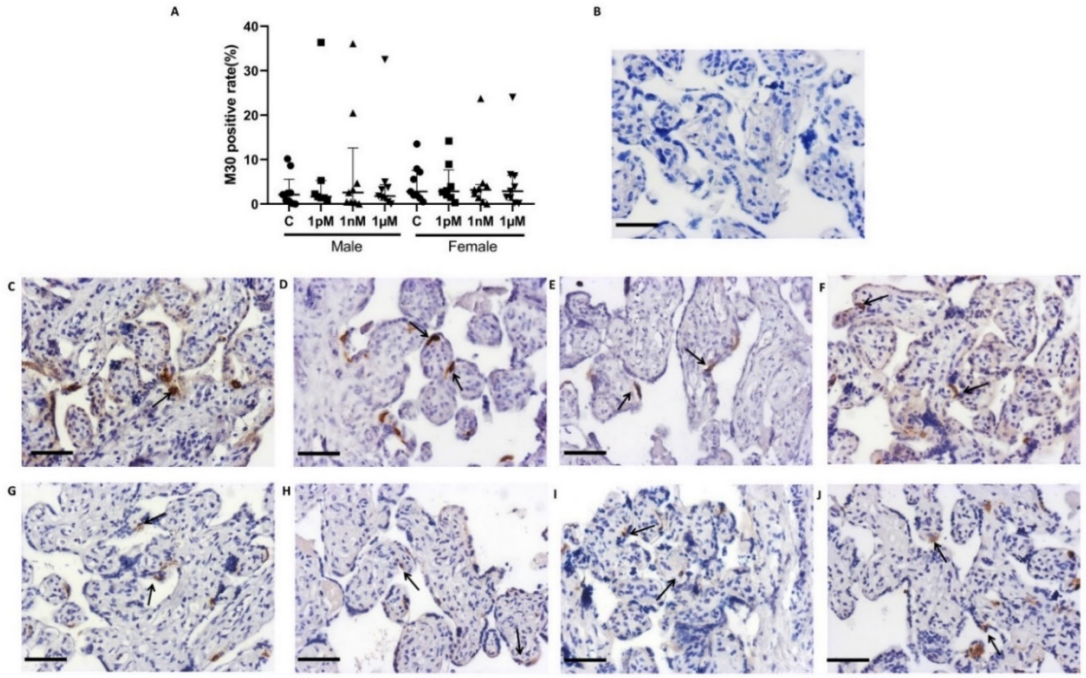

Figure 4.13 The percent of M30 positive cells in BPA treated villous explants from male and female fetuses for 24 hours of culture.

Figure 4.13A, Quantification of M30 positive cells. [B], Negative control. Representative images of immunostained placental explants from male fetuses (C-F), and female infants (G-J). Figure 4.13C and Figure 4.13G, Control (0.05% (v/v) ethanol). Figure 4.13D and Figure 4.13H, 1pM BPA. Figure 4.13E and Figure 4.13I, 1nM BPA. Figure 4.13F and Figure 4.13J, 1μM BPA. Black arrow, M30 positive cells. Kruskal-Wallis test, median+/- IQR. n=18. Bar = 50 μm.

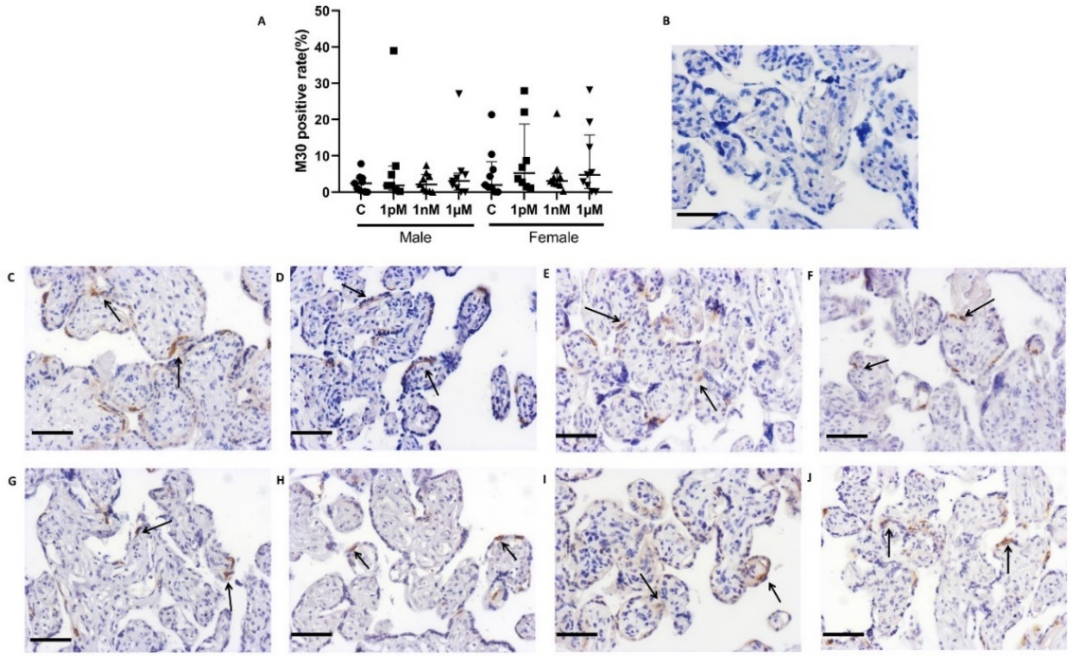

Figure 4.14 Effects of BPA on the percent of M30 positive cells in male and female placental explants for 48 hours of culture.

The quantification of M30 positive cells present in Figure 4.14A. [B], Explants were applied with mouse IgG replaced with M30 primary antibody. Representative images of immunostained placental explants from male fetuses (C-F), and female infants (G-J). Figure 4.14C and Figure 4.14G, Control (0.05% (v/v) ethanol). Figure 4.14D and Figure 4.14H, 1pM BPA. Figure 4.14E and Figure 4.14I, 1nM BPA. Figure 4.14F and Figure 4.14J, 1μM BPA. Black arrow, M30 positive cells. Kruskal-Wallis test, median+/-IQR. n=18. Bar = 50 μm.

4.4 Discussion

In this study we have explored for the first time the sex-specific effects of BPA on *ESRRG* signalling in the human term placenta. This finding provides some biological evidence to aid understanding of the differential effects of BPA exposure on male and female fetuses observed in epidemiological studies.

4.4.1 The sex-specific effects of BPA in the human placenta

BPA can target some pathways in the human trophoblastic 3A placental cell line, BeWo cells, HTR-8/SVneo cells, JEG-3 cells, human fetal oocytes, mouse placenta (ICR mice), term primary trophoblast, human term placental explants, and these pathways are related to P-glycoprotein placental transporter (encoded by *ABCB1*), oestrogen receptor, peroxisome proliferator-activator receptor gamma, protein kinase C, HSD11B2, glucose transporter-1, matrix metalloproteinases (MMP), ABCG-2 transporter protein, and mitogen-activated protein kinase (MAPK)-phosphatidylinositol3-kinases (PI3K) (Jin and Audus, 2005, Huang and Leung, 2009, Mannelli et al., 2014, Tait et al., 2015, Rajakumar et al., 2015, Ponniah et al., 2015, Lan et al., 2017, Speidel et al., 2018, Xu et al., 2019, Basak et al., 2018).

The mean BPA concentration in the fresh placental tissue was consistent with reported BPA concentrations in human placental tissues (Cao et al., 2012, Troisi et al., 2014, Lee et al., 2018, Lukasiewicz, 2020); this confirms the exogenous BPA concentrations applied in this study were physiologically relevant. We observed that short-term treatment with 1μM BPA altered *ESRRG* expression. In HeLa cells, BPA has been shown to bind to *ESRRG*, maintaining its high constitutive signalling activity without further increasing its expression (Takayanagi et al., 2006). However, our results are similar to previous studies in breast and lung cancer cell lines, which reported that both ESRRG mRNA and protein expression can be increased by short-term BPA exposure (Helies-Toussaint et al., 2014, Song et al., 2015, Zhang et al., 2016, Ryszawy et al., 2020). The only previous study to examine the relationship between *ESRRG* and BPA in trophoblast (JEG-3 cells) showed that siRNA-mediated suppression of *ESRRG* partially prevented the reduced [3H]-thymidine DNA incorporation induced by 100nM BPA treatment (Morice et al., 2011). We have confirmed the ability of BPA

to signal through *ESRRG* using a more physiological culture model, but also report sex-specific effects of BPA exposure.

The findings that 1µM BPA significantly increased ESRRG mRNA and protein expression in the female placentas but reduced expression in male placentas suggest that low dose BPA exposure can alter gene expression in the human placentas in a sex-specific manner. The concept that BPA exposure can alter gene expression is supported by data from Mao et al. who used RNA-seq to assess mouse placentas exposed to BPA during pregnancy. In these experiments BPA altered expression of 13 genes (*Actn2*, *Calm4*, *Coch*, *Cxcl14*, *Ear2/NR2F6*, *Efcab2*, *Epdr1*, *Gdf10*, *Gm9513/PATE1*, *Guca2a*, *MMP3*, *Rimklb*, *and Sfrp4*). However, these analyses did not find any sex differences (Mao et al., 2020). Numerous previous studies have used trophoblastic cell lines, including JEG-3, Bewo cells, 3A, and HTR8-SVneo cells, or primary cytotrophoblast cells to explore the effects of BPA exposure on trophoblast functions and intracellular signalling pathways (Huang and Leung, 2009, Morice et al., 2011, Wang et al., 2015, Spagnoletti et al., 2015, Rajakumar et al., 2015, Chu et al., 2018, Speidel et al., 2018, Narciso et al., 2019, Xu et al., 2019). However, as these cell lines are derived from a single placenta, and the sex of that placenta is not always specified, these studies are unable to interrogate the sex-specific effects of BPA on trophoblast function and cell signalling.

We also report that the expression of several genes downstream of *ESRRG* are altered by BPA exposure in a sex-specific manner, and responded consistently with changes in ESRRG mRNA and protein expression. Previous studies have examined the induction of *HSD11B2* mRNA expression in trophoblastic cell lines or primary cytotrophoblast following BPA exposure, without exploring the sex influences (Rajakumar et al., 2015, Basak et al., 2018). Interestingly, prenatal BPA exposure downregulated the mRNA levels of *Cyp19a1* in the brain of male offspring, whereas *Cyp19a1* mRNA was increased in the female brain, with increased anxiety-like behaviour and decreased exploratory behavior observed in both male and female F1 rats (Raja et al., 2020). The effects of BPA on *CYP191.1* mRNA expression have been reported in JEG-3 cells, a cell line derived from the placenta of a male fetus. One study showed that mRNA expression of *CYP191.1* was significantly decreased in JEG-3 cells after exposure to

5μM, 10μM or higher BPA concentrations for 24 hours (Huang and Leung, 2009); however, another study using JEG-3 cells found that both the mRNA and protein expression of CYP1A1 were significantly increased after treatment with 1μM, 10μM and 50μM BPA, but CYP191.1 mRNA and protein expression were significantly downregulated following treatment with 1, 10μM or 50μM BPA at 72 hours (Xu et al., 2019). Compared to these previous studies, we also observed reduced mRNA expression of *CYP191.1* in male placental explants at the lower BPA concentration of 1nM. Our data suggest that the sex-specific effects of BPA on *ESRRG* translate to reductions in *CYP191.1* and *HSD11B2* mRNA expression in the placentas from male infants.

To our knowledge, there are no studies that explore the expression of *HSD17B1* and *PLAC1* in the placenta following BPA exposure, and our results confirmed that these two genes can be regulated by BPA in human placentas in a sex-specific manner. Furthermore, as the sex specific effects of BPA on *ESRRG* signalling pathways were mainly observed following treatment with BPA at levels that mimic environmental exposure (1nM), this suggests that more attention should be paid to male fetuses and their placentas following maternal BPA exposure during the pregnancy. It is also important to note that BPA could target these downstream genes directly, not through ESRRG, and exert effects on other genes; further work is needed to explore these effects, to determine whether they are a direct or indirect result of BPA treatment.

4.4.2 Why might there be a difference in male and female placentas?

It is currently unclear how BPA regulates *ESRRG*-related genes in a sex-specific manner; one potential mechanism is through epigenetics, such as regulation of DNA methylation. Although the epigenetic modifications of genes in our samples are unknown, studies support the possibility that BPA treatment can lead to epigenetic modifications in the liver and brain of mice (Raja et al., 2020). Maternal BPA exposure reduced the mRNA expression of *Dnmt3b* and *Kmt2c* in the livers of male offspring on postnatal day 1, but significantly increased the expression of *Kmt2c* in the livers of in female offspring, due to epigenetic modifications (Strakovsky et al., 2015). Another study also found that BPA could modulate the expression of genes within the oestrogen receptor (ER) signalling pathway, including *Esrrg*, in a sex-specific manner

in the brains of mice; this was mediated by epigenetic modifications (Kundakovic et al., 2013).

BPA exposure has also been shown to alter DNA methylation status in the placentas of human and mice, and in the first-trimester trophoblast cell line, HTR8/SVneo, a sex-unspecified cell line; affected genes included those which regulate proliferation and migration, such as *LINE-1*, *HLA-DRB6*, *HDAC4*, *HLA-DRB1*, and *WNT-2* (Susiarjo et al., 2013, Nahar et al., 2015, Basak et al., 2018, Ye et al., 2019, Song et al., 2021), but only a few studies have explored sex-specific differences in placental gene methylation (Vilahur et al., 2014, Song et al., 2021). Song et al. showed there was no sex association between BPA exposure and the hypermethylation of *HLA-DRB6* in the human placenta, but only examined 6 placentas with (n=3) or without (n=3) detectable BPA concentrations (Song et al., 2021). However, Vilahur et al. studied 192 placentas and found that the total effective xenoestrogen burden (TEXB) biomarker, TEXB-alpha, correlated with decreased AluYb8 DNA methylation in male placentas, but not in female placentas (Vilahur et al., 2014), providing more evidence that maternal BPA exposure may alter DNA methylation in human placentas in a sex-specific manner.

BPA (1nM) has also been reported to decrease the CpG methylation of several gene promoters associated with metabolic and oxidative stress in the HTR8/SVneo cell line, including *GSR*, *DNAJA1*, *PRDX2*, *GPX3*, *DDIT3*, *HERPUD1*, *INSIG1* and *MBTPS1* (Basak et al., 2018). Given that *ESRRG* plays an important role in regulating placental metabolism, mitochondrial function, and oxidative stress in the cytotrophoblast (Poidatz et al., 2012), altered methylation of promotors within the *ESRRG* gene might be one of the mechanisms by which BPA exerts its sex- specific effects (Figure 4.15).

Another possible explanation for our findings may be due to differences in BPA metabolism between male and female placentas. BPA is metabolized by UDP-glucuronosyltransferase, which is expressed in the human kidney, liver and placenta; all *UGT2B* isoforms and *UGT1A* are expressed in the human placenta (Collier et al., 2002, Reimers et al., 2011). Several studies have reported that blood BPA concentration is higher in males than females, both in mice and humans (Takeuchi et al., 2004), which correlates with the observation that *Ugtb1* is more highly expressed in the livers of female mice, compared to males (Shibata et al., 2002, Takeuchi et al.,

2004). Furthermore, the level of circulating BPA metabolites is higher in women than men (Mazur et al., 2010), suggesting that elevated UGT1A expression leads to more rapid BPA metabolism in females. Sex-specific gene expression of UGT has been reported in the kidney and liver of mice, but was not observed in the placenta (Buckley and Klaassen, 2007). To our knowledge, sex differences in UGT expression in the human placenta have not been well studied. Further studies exploring the placental UGT expression might be helpful to explain the sex-specific effects observed in our study.

4.4.3 How does this help our understanding of the pathogenesis of fetal growth restriction in human pregnancy?

Our ultimate aim is to understand the mechanisms underlying placental dysfunction observed in FGR, with a particular focus on the role of *ESRRG* (Zou et al., 2021). The sex-specific effects of BPA on *ESRRG* signalling we observed might begin to provide a biological explanation for the observed relationship between low fetal weight in male neonates and maternal BPA exposure seen in epidemiological studies. Even though the relationship between fetal weight and maternal BPA exposure has been considered controversial by some (Miao et al., 2011, Chou et al., 2011, Philippat et al., 2012), there are other studies which show a significant relationship between low fetal weight and BPA exposure in both mice and humans (Chou et al., 2011, Miao et al., 2011, Muller et al., 2018). In mice, prenatal BPA exposure can cause FGR, but sex differences were not explored (Muller et al., 2018). In human studies, maternal BPA exposure increased the risk of reduced fetal weight and SGA in male infants (Chou et al., 2011, Miao et al., 2011). As *ESRRG* is significantly decreased in human placentas from FGR pregnancies, and reduced expression of *ESRRG* is associated with abnormal proliferation and invasion of first-trimester trophoblastic cell lines (Zhu et al., 2018a, Zou et al., 2019), compounds which decrease *ESRRG* expression, such as BPA, may act through *ESRRG* to induce placental dysfunction and FGR. Furthermore, reduced fetal weight in male neonates following maternal BPA exposure might be a consequence of reduced placental expression and impaired signalling through *ESRRG* (Figure 4.15). Our results suggest that male placentas may be more vulnerable to maternal BPA exposure than female placentas. Although no changes in the percentage of Ki67

positive cells and M30 positive cells were observed following short term BPA exposure in this study, previous studies have described anti-proliferative and apoptotic effects of BPA on term primary cytotrophoblast or BeWo cells, by measuring [^{3}H]-thymidine incorporation or quantifying changes in apoptotic markers, including M30 and tumor-necrosis factor alpha (TNF-alpha) protein (Benachour and Aris, 2009, Morice et al., 2011). In this study, we quantified Ki67 protein expression using immunostaining, which is a proliferative marker that is expressed in all active phases of the cell cycle (G1, S, G2 and M); we also used an anti-M30 antibody, which is directed against a specific epitope of cytokeratin 18 (CK18) that is formed by early caspase cleavage in apoptotic cells (Scholzen and Gerdes, 2000, Kharfi et al., 2006). Additional markers of apoptosis and proliferation should be explored to further investigate the effects of BPA on trophoblast turnover in cultured explants.

The increase in hCG secretion following 1μM BPA treatment is consistent with previous studies, which found BPA can induce the secretion and mRNA expression of hCG in term primary trophoblast and BeWo cells (Mannelli et al., 2014, Rajakumar et al., 2015, Narciso et al., 2019), and first trimester villous explants (Morck et al., 2010). More studies are needed to confirm the relationship between trophoblast differentiation, hCG secretion and BPA-induced ESRRG signalling. In contrast, BPA did not alter LDH release from explants at 1nM or 1μM, after 24 or 48 hours of culture; however, BPA increased LDH release from explants treated with 1pM BPA for 24 hours. Prior studies have indicated that BPA exposures between the ranges of 100pM to 50μM did not alter LDH levels in culture medium of JEG-3 cells (Morice et al., 2011, Xu et al., 2019). Interestingly, the JEG-3 cell line is derived from a male placenta, and the high LDH release we observed mainly occurred in BPA-exposed female placentas. These data might suggest that female placentas may be more sensitive to low dose BPA exposure than male placentas.

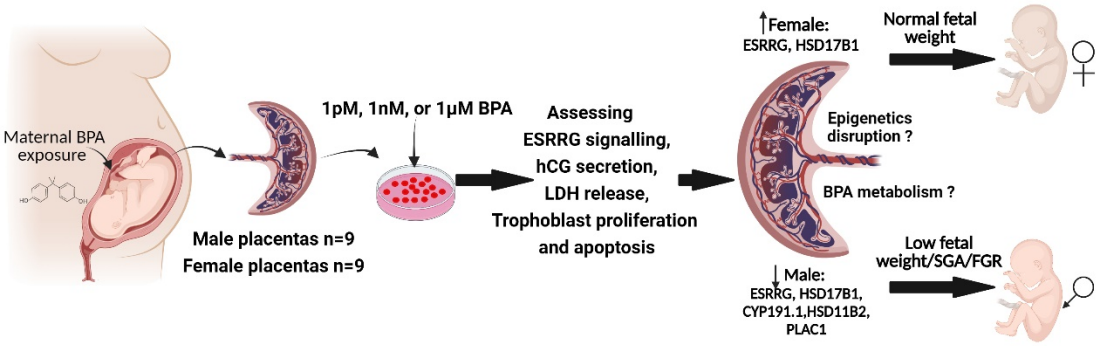

Figure 4.15 Schematic illustration of the ESRRG signalling pathways mediated by BPA in a sex-specific manner.

4.4.4 Strengths and limitations of this study

This study is strengthened by the use of a relevant range of BPA concentrations, which included those reflecting environmental exposure, in a controlled experimental protocol using BPA-free reagents and plasticware. The use of fresh term placental villous explants, as opposed to transformed cell lines or those derived from neoplasias, represents the most physiologically appropriate ex vivo culture model available and allows comparison to be made with other studies which used this approach. Nevertheless, there are several limitations in this study, including the relatively small sample size. In this study, there was some variability in hCG secretion, LDH release, and the percentage of Ki-67 or M30 positive cells, reflecting inherent differences in the experimental tissues. However, all reported values are within the ranges reported by our previous studies or the data from others (Turner et al., 2006, Moll et al., 2007, King et al., 2016, Renshall et al., 2021). Nevertheless, our study recruited 18 placentas for villous explant culture; a further study with a larger sample size may be needed to identify potential BPA effects on trophoblast cell turnover using additional proliferative and apoptotic markers, particularly if the influence of maternal plasma BPA levels during pregnancy are also to be taken into account. It would also be worthwhile to further explore the non-monotonic response to BPA in the explant model by testing a wider range of physiologically relevant BPA concentrations. Furthermore, the influence of BPA exposure on other aspects of placental function e.g. hormone secretion and nutrient transport, and BPA-effects on trophoblast turnover in early pregnancy, will also be important areas for study. Finally, the effects of chronic, rather than acute BPA exposure may more accurately represent environmental conditions to which the placenta is exposed in utero. The cultured explants model may also affect the exploration of ESRRG signaling pathways as this model present a low protein expression of ESRRG signalling pathways and the different ESRRG protein localisation has been found between fresh placental tissues and cultured villous explants. Future work will be explored ESRRG signaling pathways *in vitro* and *in vivo* models, such as organoids.

4.5 Conclusion:

In summary, we demonstrated that BPA exposure altered *ESRRG* signalling pathways in a sex-specific manner in human placental explants, but did not affect the basal level of hCG or LDH secretion, nor the number of placental cells in cycle or undergoing apoptosis. As BPA exposure alters *ESRRG* expression, and *ESRRG* regulates many aspects of trophoblast function, BPA may act through *ESRRG* to induce or enhance placental dysfunction and contribute to the pathophysiology of FGR.

4.6 Acknowledgments

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5 CHAPTER 5: miR-377 Downregulates *ESRRG* Signalling in the Placenta and Disrupts Cell Turnover in Term Placental Explants

Zhiyong Zou, Karen Forbes, Lynda K Harris, Alexander E P Heazell

Abstract

Oestrogen related receptor gamma (*ESRRG*), a nuclear receptor, is significantly decreased in placentas complicated with fetal growth restriction (FGR) and might be causally related to the observed placental dysfunction. miR-377 is predicted to be an upstream regulator of *ESSRG* and is associated with impaired cytotrophoblast proliferation in cultured first trimester villous explants. We hypothesised that increasing miR-377 expression would reduce expression of *ESRRG* and lead to abnormal cell turnover.

Term placental explants (n=7) from appropriate for gestational age (AGA) infants were cultured for 48 hours with miR-377 mimics, or non-targeting controls. Levels of miR-377, *ESRRG* and its downstream genes were assessed by real-time QPCR. Expression and localisation of *ESRRG,* and the numbers of apoptotic and proliferating cells were assessed by immunohistochemistry for cytokeratin M30 and Ki67 respectively.

miR-377 was increased in the cultured explants transfected with 100nM miR-377 mimics (Median ± IQR, miR-377 mimic (normalized to control group), 1.4±2.6fold, $P<0.05$). ESRRG mRNA (Median ± IQR, miR-377 mimic (normalized to control group), 0.7±0.4fold) and protein expression (Median ± IQR, miR-377 mimic (normalized to control group), 0.2±0.5fold) were significantly decreased in explants transfected with the miR-377 mimic ($P<0.05$), but downstream targets of *ESRRG*, *HSD17B1*, *CYP191.1*, *HSD11B2*, and *PLAC1* were not reduced. The miR-377 mimic (100nM) reduced the number of cells in cycle (Median ± IQR, 0.9±0.5% (control) vs 0.1±0.1% (miR-377 mimic)) and increased apoptosis (Median ± IQR, 0.5±0.4% (control) vs 1.2±1.3% (miR-377 mimic)) in the cultured explants. These data suggest that miR-377 could alter cell turnover in term placental tissue by regulating the expression of *ESRRG*. As downstream targets of *ESRRG* were not altered, further studies are needed to determine disruption of this pathway affects cell turnover, or whether miR-377 mediates these effects through another pathway.

5.4Introduction

Fetal growth restriction (FGR) describes a condition where the fetus does not achieve its optimal growth potential in-utero. It affects 10% of pregnancies and is a major risk factor for stillbirth, neonatal death, neurodevelopmental delay in childhood, and cardiovascular and metabolic disorders in adulthood (Baschat, 2011, Gardosi et al., 2013, Crispi et al., 2018). There is no effective therapeutic drug to manage FGR; the only intervention is early delivery directed by fetal surveillance. Understanding the mechanisms responsible for the development of FGR may lead to the identification of novel therapeutic targets. Placental dysfunction (as evidenced by reduced cell proliferation and induced cell apoptosis) is the main cause of FGR, and some studies suggest that altered oestrogen related receptor gamma (*ESRRG*) signalling might contribute to this (Heazell et al., 2011, Poidatz et al., 2015a, Zhu et al., 2018a).

ESRRG, a nuclear receptor, is highly expressed in the trophoblast in normal term placenta but is significantly decreased in FGR placentas (Poidatz et al., 2015a, Zhu et al., 2018a). Previous studies have demonstrated that *ESRRG* can modulate trophoblast proliferation, apoptosis and mitochondrial function, although these were mainly conducted in the trophoblast-like cell line HTR-8/SVneo (Poidatz et al., 2012, Poidatz et al., 2015b, Zhu et al., 2018a). *ESRRG* activates a variety of downstream genes, including hydroxysteroid 17-beta dehydrogenase 1 (*HSD17B1*), cytochrome P-450 (*CYP191.1*), placenta-specific protein 1 (*PLAC1*), and hydroxysteroid 11-beta dehydrogenase 2 (*HSD11B2)* (Kumar and Mendelson, 2011, Luo et al., 2013, Zhu et al., 2018a), and these downstream genes may be related to development of FGR (Shams et al., 1998, Tzschoppe et al., 2009, Audette et al., 2010, Chang et al., 2016, Zhu et al., 2018a, Sifakis et al., 2018, Wan et al., 2019, Anelli et al., 2019, Devor et al., 2021). Recently, we have demonstrated that expression of *ESRRG* and its downstream genes are altered in response to hypoxia or exposure to BPA (Chapter 3 and Chapter 4); both of these factors reduce fetal growth (Tal et al., 2010, Chou et al., 2011, Miao et al., 2011, Stubert et al., 2012, Snijder et al., 2013, Robb et al., 2017) further supporting the role for *ESRRG* in the pathogenesis of FGR. We and others have identified microRNAs (miRNAs) that are implicated in the pathogenesis of FGR and regulate placental growth (Forbes et al., 2012, Farrokhnia et al., 2014, Baker et al., 2021,

Quilang et al., 2021); we have also demonstrated that miRNA-based drugs have the potential to improve placental and fetal growth *in vitro* and *in vivo* (Beards et al., 2017).

miRNAs are small non-coding RNAs which post-transcriptionally repress downstream effectors by degradation of target messenger RNA (mRNA) and/or repressing translation (Bartel, 2004, Selbach et al., 2008). Abnormal expression of some miRNAs in maternal serum or placental tissues contributes to the development of pregnancy complications such as FGR and preeclampsia (Anton et al., 2013, Whitehead et al., 2013, Mouillet et al., 2015, Baker et al., 2021). Although several studies have identified placental miRNAs that regulate *ESRRG* (Gao et al., 2018, Liu et al., 2018, Zou et al., 2019), these studies were conducted using trophoblast-like cell lines, HTR-8/Svneo cells. Hence, there is a need to repeat this work in a more physiological, human placental explant model to find other novels regulatory miRNAs, and explore how altered miRNA and *ESRRG* expression is linked to placental dysfunction. Identification of *ESRRG* regulatory miRNAs may inform the development of therapeutics to prevent the placental dysfunction underlying FGR pregnancies.

Therefore, our current study will use a placental explant model to test potential miRNA regulators of *ESRRG* identified through a structured search and to determine the relationship between expression of these miRNAs, *ESRRG* and abnormal trophoblast turnover.

5.2 Materials and Methods

5.2.1 Materials

All chemicals were obtained from Sigma-Aldrich (Sigma, UK) unless otherwise mentioned.

5.2.2 miRNAs prediction

We used three biogenetic software packages TargetScan (http://www.targetscan.org/vert_80/), miRanda (http://mirdb.org/) and Pictar (https://pictar.mdc-berlin.de/), accessed on November 9th of 2021) to predict the upstream miRNAs for 3'-UTR of *ESRRG.* ESRRG was entered on the website of TargetScan; human species and the conserved miRNAs families in the 3'-UTR were selected. Then I searched in PubMed for all miRNAs on the list of TargetScan and

screened whether these miRNAs were altered in placentas from women with FGR or pre-eclampsia or were related to modulation of human trophoblast function were selected for investigation in this study. I also searched whether these miRNAs can be found in miRanda and Pictar as upstream miRNAs of ESRRG.

5.2.3 Placental tissue collection

The archived first trimester placental explants were from a previous study which has been approved by the Local Research Ethics Committee (Ref: 13/NW/0205; 08/H1010/55(+5)) with written informed consent, and all tissue was obtained from Saint Mary's Hospital, Manchester (Farrokhnia et al., 2014). Demographic information of the archived first trimester placental tissue used in this study has been described previously (Farrokhnia et al., 2014).

For the term placental tissue, written informed consent was obtained from all patients in Saint Mary's Hospital, Manchester, and this study was approved by the Research Ethics Committee (08/H1010/55+5). Term placental tissue was collected within 30 minutes of delivery from appropriate for gestational age (AGA) pregnancies (individualised birth weight ratios (IBR) between the 10^{th} to 90^{th} centile; n=9) or FGR pregnancies (IBR below the 5^{th} centile; n=9) infants. The exclusion criteria included pregnancies complicated with chronic hypertension, preeclampsia, renal disease, diabetes, gestational diabetes, premature rupture of membranes, or collagen vascular disease, and fetuses complicated with fetal anomalies or chromosomal abnormalities. Fresh placental tissue from uncomplicated pregnancies (n=7) was collected for explant culture. The demographic information of women donating FGR, and AGA placentas is listed in Supplementary Table 5.1; the demographic details of the women donating term AGA placentas used for explant cultures is listed in Table 5.1.

5.2.4 Placental explant model

Placental explants were prepared as previously described (Heazell et al., 2007, Heazell et al., 2008). After randomly selecting six different sites in the placenta, excluding sites of necrosis, infarction, and excessive tears, six chunks of villous tissue ($2cm^3$) were aseptically excised and placed into warm, sterile PBS in the tissue culture hood. Villous explants were dissected further in a petri dish and washed in warm PBS twice to

remove the excess blood. The size of each placental explant was approximately 2mm^3. Sterile netwells (Corning Inc, NY, USA) were inserted into 12-well plates and 1:1 of Dulbecco's modified Eagle medium (DMEM; Gibco, UK)/Ham's F12 (Gibco, UK) supplemented with penicillin (0.6mg/l), streptomycin (100µg/ml), L-glutamine (0.292g/l), and 10% fetal calf serum (Gibco, UK) was added. Three villous tissue fragments were pooled from 3 different sample sites were added to each netwell.

5.2.5 Transfection with DharmaFECT transfection reagent

The transfection method used the lipid carrier, DharmaFECT 2 (Dharmacon, GE Healthcare, UK), with miR-377 mimics or a non-targeting control (Dharmacon, lnc., USA) to transfect BeWo cells and placental explants, as described in Section 2.2.4 and Section 2.3, and according to a previous study (Forbes et al., 2009).

5.2.6 Overexpression of miR-377

When the explant cultures were set up, miR-377 mimics or a non-targeting control (Dharmacon, lnc., USA) were added directly to the explant culture media (final concentration 10nM-100nM), as previously described (Forbes et al., 2009). Explants were cultured at 21% O_2 (with 5% CO_2) for 48 hours before explants and media were collected and processed for downstream applications. The transfection method for archived first trimester explants is described in a previous study (Farrokhnia et al., 2014).

5.2.7 Reverse transcription-polymerase chain reaction (RT-PCR)

Following culture, villous explants were stored in RNA-later at 4 °C overnight and then stored at -80 °C immediately, until RNA extraction was performed. Total RNA extraction was performed using miRNeasy mini kit (QIAGEN, UK) following the manufacturer's instructions. A Nanodrop (Thermo Fisher Scientific, USA) was used to assess the concentration and quality of the total RNA. *mRNA:* For normal mRNA, a total of 2500ng mRNA from each sample was converted to cDNA using an AffinityScript Multiple Temperature cDNA Synthesis kit (Agilent, USA) according to the cDNA synthesis protocol. The sequences of PCR primers for *ESRRG*, its downstream genes, and *RPLP0* (house-keeping gene, 60S acidic ribosomal protein P0, Eurofins, UK). The powerup SYBR Green Master Mix (Thermo Fisher Scientific, USA) was used in the PCR

reaction and the Applied Biosystems Step-one system (Thermo Fisher Scientific, USA) was used to run the reaction with an annealing temperature of 60°C and 40 cycles followed by a melt curve step. *miRNA*: a miRCURY LNA RT kit was used for reverse transcription of miRNAs and total RNA (10ng) was applied according to the quick-start protocol. The sequences of PCR primers for miRNAs, and endogenous reference genes, U6 snRNA (Dharmacon, lnc., USA) are listed in Supplementary Table 5.2. U6 was expressed stably in FGR and normal placentas and was used to normalise the miRNA expression (Supplementary Figure 1). The miRCURY LNA SYBR Green PCR kit (QIAGEN, Germany) was used to measure miRNA expression, using an annealing temperature of 56°C and 40 cycles, followed by a melt curve step according to the manufacturer's protocols. Data are presented as relative expression to the housekeeping gene U6, calculated by the $2^{-\triangle\triangle CT}$ method.

5.2.8 Immunohistochemistry

Following culture, placental explants were harvested and fixed in 4% neutral buffered formalin overnight at 4°C. Archived first-trimester placental wax blocks were also analysed for comparison. 5µm sections were dewaxed and rehydrated, then antigen retrieval was performed by microwave boiling for 5 minutes twice at full power (800w) in 0.01M citrate buffer (pH 6.0). 3% (v/v) hydrogen peroxide was applied for 10 minutes to block endogenous peroxidase activity. Sections were incubated with non-immune block (10% goat serum and 2 % human serum in 0.1% TBST (TBS-Tween-20) for 30 minutes at room temperature, and then incubated with a polyclonal antibody against ESRRG (Abcam 219547, 10µg/ml), a monoclonal antibody against Ki-67 (Dako, 0.174µg/ml), or a monoclonal antibody against M30 (Roche, 0.132ng/ul) overnight at 4 °C. Ki-67 positive staining identifies the number of cells in cycle and is a marker for cell proliferation (Heazell et al., 2008, Baker et al., 2017, Renshall et al., 2021). M30 positive staining is a marker for apoptosis (Baker et al., 2017, Renshall et al., 2021). Isotype-specific non-immune rabbit or mouse IgG was used as a negative control and was applied at the same concentration as the primary antibodies. Sections were washed in TBS and 0.6% TBST (TBS-Tween-20). After incubating with the appropriate secondary antibody (biotin conjugated goat anti-mouse or anti-rabbit antibodies, Dako-Cytomation, UK, 3.85 µg/ml) for 30 minutes at room temperature, avidin-

peroxidase (5µg/ml) was applied to the sections for 30 minutes. The chromogenic substrate diaminobenzidine (DAB; Sigma-Aldrich, UK) was applied to the sections for between 2-10 minutes and colour development was monitored under the microscope. All slides were counterstained with Harris's hematoxylin (Sigma-Aldrich, UK) for 5minutes. The sections were then dehydrated and mounted in DPX (Sigma-Aldrich, UK). QuPath version 0.2.3 (developed by the University of Edinburgh) was used to analyse the IHC staining results (Bankhead et al., 2017); the number of DAB positive cells were quantified in relation to the total number of cell nuclei to provide a percentage of stained cells, and the mean proportion was calculated to present as the proliferation index or apoptotic index. Quantification of ESRRG was achieved by quantifying the DAB positive area and the total tissue area using QuPath.

5.2.9 Analysis of human chorionic gonadotropin (hCG) secretion and lactate dehydrogenase (LDH)

hCG secretion, a marker of cytotrophoblast differentiation, was measured in explant-conditioned culture medium by ELISA (DRG Diagnostics, Marburg, Germany). A cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany) was used to measure lactate dehydrogenase (LDH) release in the culture medium according to the manufacturer's instructions. Placental explants, which were harvested at 48h and lysed in 0.3M NaOH, were used to provide a value for total protein content, as measured by using a standard BioRad protein assay (Bio-Rad Laboratories, Hempstead, UK). These data were used for normalization of hCG secretion and LDH release.

5.2.10 Statistical analysis

Data were presented as mean ± standard deviation (SD) (normally distributed) or median+/-interquartile range (IQR) (not normally distributed). Statistical analysis was performed by using GraphPad Prism version 7.0 (GraphPad Software, USA). Data were analysed by t-test (unpaired or paired) or ANOVA (parametric) or Mann Whitney U test or Kruskal Wallis test (non-parametric). A Friedman multiple comparison test was conducted to analyse paired data depending on the normal distribution. The threshold of statistical significance was set at $P<0.05$.

5.3 Results

5.3.1 Predicted miRNA regulators of *ESRRG*

After reviewing previous studies undertaken using human placental tissue or trophoblast-like cell lines (Choi et al., 2013, Farrokhnia et al., 2014, Xu et al., 2014, Castro-Villegas et al., 2015, Hromadnikova et al., 2015a, Baker et al., 2017, Hromadnikova et al., 2017, Niu et al., 2018, Muller-Deile et al., 2018, Yang et al., 2018, Huang et al., 2019a, Huang et al., 2019b, Yang et al., 2019, Yu et al., 2019, Inno et al., 2021), we identified 7 miRNAs which may be upstream regulators of *ESRRG*, including miR-204-5p, miR-377, miR-30a, miR-26a, miR-23-3p, miR-34b, and miR-30e. Biogenetics software such as TargetScan was used to confirm the presence of a putative *ESRRG* binding site within each miRNA, the details of which are listed in Table 5.2.

As the aim of this study was to explore whether miRNAs upstream of *ESRRG* can regulate placental cell turnover (including cytotrophoblast proliferation and apoptosis), and as miR-377 was the only miRNA identified that had previously been shown to modulate cytotrophoblast proliferation (Farrokhnia et al., 2014), we assessed whether miR-377 could signal through *ESRRG* to regulate placental cell turnover.

5.3.2 Transfection with miR-377 mimics in BeWo cells

BeWo cells were transfected with miR-377 mimics or a nontargeting control (40nM) with transfection reagent; the data is shown in Supplementary Figure 5.2. Median mRNA levels of *ESRRG* were comparable between control groups (untreated control, or non-targeting control (40nM)) and BeWo cells transfected with miR-377 mimics (40nM) for 24 to 72 hours (Supplementary figure 5.2A). *ESRRG* mRNA expression was induced in the BeWo cells which were treated with transfection reagent (Mock) compared to untreated control for 48 hours (Supplementary Figure 5.2C). But miR-377 RNA levels were significantly increased in the BeWo cells transfected with 40nM miR-377 mimics compared to untreated control or non-targeting control (40nM), which suggested miR-377 mimics increased miR-377 RNA levels in the BeWo cells (Supplementary figure 5.2B).

5.3.3 Overexpression of miR-377 reduced the expression of *ESRRG* in term villous explants

As attempted transfection with miR-377 mimics in BeWo cells did not produce the anticipated effects on ESRRG we then explored modulation of miR-377 in ex vivo placental explants where the pathway appears intact. Firstly, immunostaining with an antibody against ESRRG was undertaken in archived first trimester placental explants transfected with miR-377 mimics (100nM) from a previous project (Farrokhnia et al., 2014), this showed ESRRG protein expression within the syncytium was dramatically reduced, compared to levels in untreated control explants, or explants treated with a non-targeting control miRNA (100nM) (Figure 5.1). These data suggested the potential regulation between miR-377 and ESRRG was intact in cultured first trimester placental explants.

Term placental explants were transfected with miR-377 mimics or non-targeting mimics (26.7nM) with transfection reagent for 48 hours and the results suggested that miR-377 mimics (26.7nM) did not alter ESRRG mRNA and protein expression (Supplementary Figure 5.3A and B) and mRNA levels of *HSD11B2*, and *HSD17B1*, which were downstream effectors of *ESRRG* (Supplementary Figure 5.3C and D).

In addition, term placental explants were incubated with miR-377 mimics (10-100nM) or non-targeting control (10-100nM) in the absence of transfection reagent for 48h. miR-377 mimics (10nM, 20nM, and 50nM) and all concentrations of non-targeting control (10nM-100nM) did not significantly alter miR-377 RNA levels or ESRRG's mRNA and protein expression (Figure 5.2A).

In term villous explants, miR-377 overexpression was achieved using a concentration of 100nM of the mimic which resulted in a 41.4% increase (1.4 ± 2.6-fold; median ± IQR) in expression of mimic, compared to the non-targeting control (Figure 5.2A). *ESRRG* mRNA (Figure 5.2B) and protein expression (Figure 5.2C&D) were significantly decreased following miR-377 overexpression in term placental explants. mRNA: median ± IQR, 0.7±0.4, P=0.02, 30% reduction vs non-targeting control, n=7; protein: median ± IQR, 0.2±0.5, P=0.02, 74% reduction vs non-targeting control (Figure5.2B and 5.2C).

Table 5.1 Participant demographics of placental tissue used for cultured explants

Characteristic	Healthy pregnancies (n=7)
Birthweight (g)	3470.7±410.7(2866-4162)
Birth centile	65.6±26.8 (11.7-98.9)
Fetal sex (female/male)	3/4
Gestational age at delivery (weeks)	39.0±0.6 (37.4-39.3)
Maternal age (years)	32.8±2.9 (26.0-39.0)
Parity	1±1 (0-4)
Gravidity	2±1 (1-5)
Nulliparous pregnant (%)	2 (28.6%)
Ethnicity	
White British	3 (42.9%)
Pakistani	1 (14.3%)
Croatian	1 (14.3%)
Irish	1 (14.3%)
Others	1 (14.3%)
BMI (kg/m^2)	24.2±2.6 (19.0-27.2)

The result was shown as mean ± SD or median ± IQR, NS, no significant, BMI, body mass index.

Table 5.2 The details of the predictive microRNAs

Name	Target-scan		miRanda	Pictar	Studies in human Placentas or cell lines
	Position in 3'-UTR	Seed match	Target score	score	
miR-204-5p	39-46	8mer	95		miR-204-5p mimics repressed the invasion of bewo cells and JEG-3 cells via modulating matrix metalloproteinase-9 (MMP9)(Yu et al., 2015).
miR-377-3p	3129-3135	7mer-m8			Higher level in 3rd trimester placentas and overexpression of miR-377 in first trimester placental explants significantly reduced basal cytotrophoblast proliferation (Farrokhnia et al., 2014).
miR-30a-5p	3303-3309	7mer-m8			Placental expression of miR-30a-5p is related to pregnancies complicated by preeclampsia (Inno et al., 2021), and reduced miR-30a-5p signalling pathways is associated with induced autophagy in mice placentas(Huang et al., 2019b).
miR-26a-5p	2534-2540	7mer-1A		0.75	miR-26a-5p was significantly induced in the placentas complicated by preeclampsia (Muller-

				Deile et al., 2018), but another two studies observed a down-regulation of placental miR-26a-5p in preeclampsia pregnancies (Hromadnikova et al., 2015a, Hromadnikova et al., 2017). Overexpression of miR-26a-5p reduce the migration and invasion of HTR-8Svneo cell line (Yang et al., 2019).
miR-23-(3p)	1378-1384	7mer-m8	86	The invasion of HTR-8Svneo was reduced in the cells with miR-23a-3p mimics (Yang et al., 2018).
	1788-1794	7mer-1A		
	3027-3034	8mer		
miR-34b-5p	1386-1392	7mer-m8	64	Placental expression of miR-34b-5p was upregulated in low maternal folate status (Baker et al., 2017).
miR-30e-3p	2807-2814	8mer	53	miR-30e may play an important role in creating a micro-immune tolerance environment of maternal-fetal interface (Huang et al., 2019a).

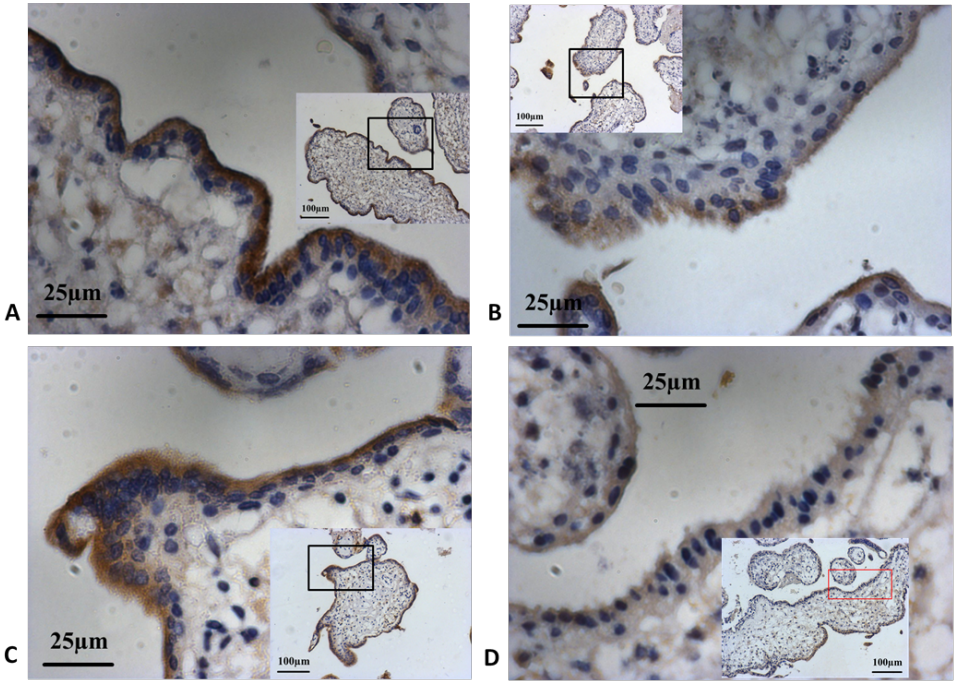


Figure 5.1 IHC staining of ESRRG expression in first-trimester placental villous explants transfected with miR-377 mimics with transfection reagents.

[A], untreated control; [B], mock; [C], explants transfected with 100nM pre-miR control; [D], the villous explants transfected with 100nM miR-377 mimics; n=1.

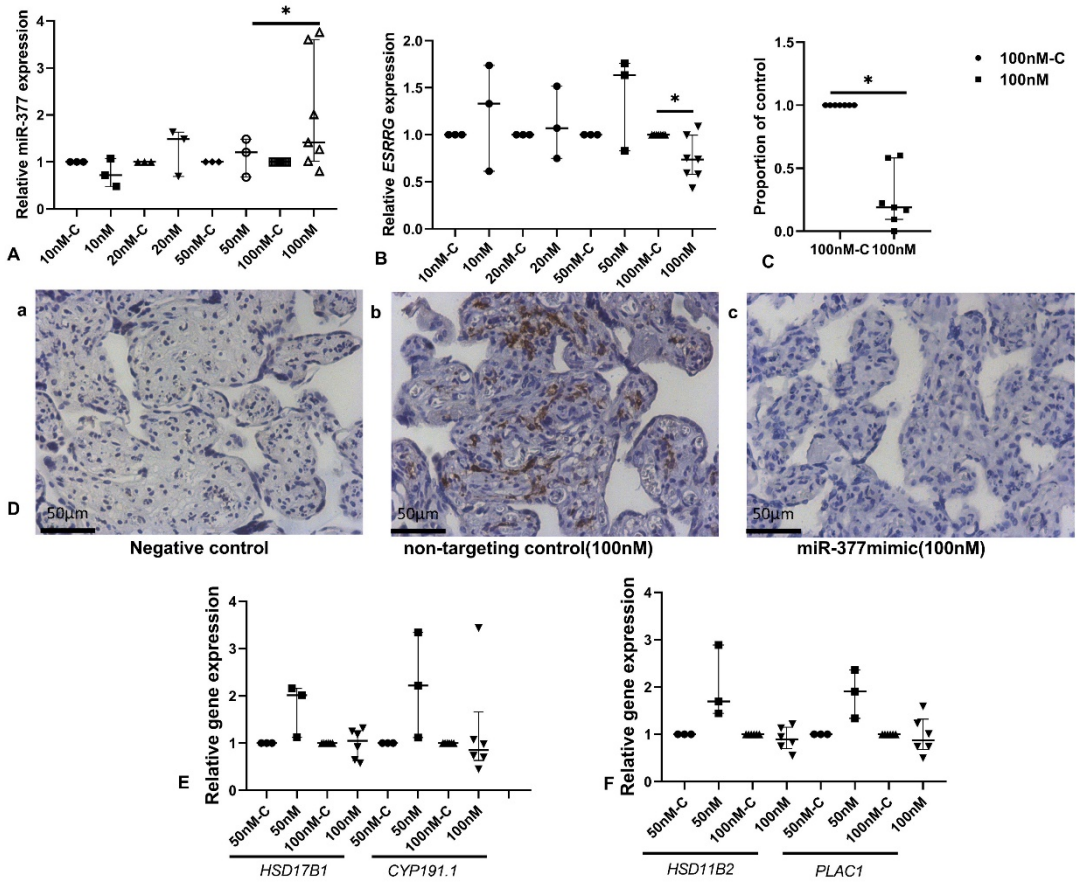


Figure 5.2 The expression of miR-377-ESRRG signalling pathways in the term villous explants incubated with 10nM to 100nM miR-377 mimics or non-targeting control for 48 hours.

[A] miR-377 expression. [B] mRNA expression of *ESRRG*. [C] Quantification of ESRRG protein staining. [D] Representative images of ESRRG protein staining in the explants transfected with miR-377 mimics (b), or non-targeting control (c); negative control with the non-immune rabbit IgG (a). [E] and [F], mRNA levels of *ESRRG*'s downstream genes. *HSD17B1*, hydroxysteroid 17-beta dehydrogenase 1; *CYP191.1*, cytochrome P-450; *PLAC1*, placenta-specific 1; *HSD11B2*, hydroxysteroid 11-beta dehydrogenase 2. Median+/-IQR; one sample Wilcoxon test. *, P<0.05.

5.3.4 Overexpression of miR-377 did not alter expression of genes downstream of *ESRRG* in term villous explants

The mRNA levels of several genes downstream of *ESRRG* did not change in the explants incubated with miR-377 mimics, when compared to levels in explants treated with the non-targeting control miRNA (Figure 5.2E and 5.2F). These included *HSD17B1*: median ± IQR, 1.0 ± 0.6; *CYP191.1*: median ± IQR, 0.9 ±1.0; *HSD11B2*: median ± IQR, 0.9±0.5; *PLAC1*: median ± IQR, 0.9±0.6.

5.3.5 Overexpression of miR-377 did not alter placental hormone secretion

Overexpression of miR-377 in term placental explants did not alter basal levels of hCG secretion or LDH release into the culture medium when compared to levels in explants treated with the non-targeting control miRNA (Figure 5.3A and Figure 5.3B).

5.3.6 Overexpression of miR-377 altered trophoblast cell turnover

Overexpression of miR-377 in term placental explants reduced the number of cells in cycle (miR-377 mimic vs control, median ± IQR, 0.1±0.13% vs 0.9±0.5%, Figure 5.4A, 49.9% reduction in Ki-67 positive cells, P=0.008) and increased the number of cells undergoing apoptosis (miR-377 mimic vs control, median ± IQR, 1.2±1.3% vs 0.5±0.5%, Figure 5.4B, 2.5-fold induction of M30 positive cells, P=0.02), when compared to explants treated with the non-targeting control miRNA.

5.3.7 Expression of predicted miRNA regulators of *ESRRG* in AGA and FGR placentas

The expression level of the seven potential upstream regulators of *ESRRG* (miR-204-5p, miR-377, miR-30a, miR-26a, miR-23-3p, miR-34b, and miR-30e) was similar in FGR and AGA placentas (Supplementary Figure 5.4) and was independent of fetal sex (Supplementary Figure 5.5).

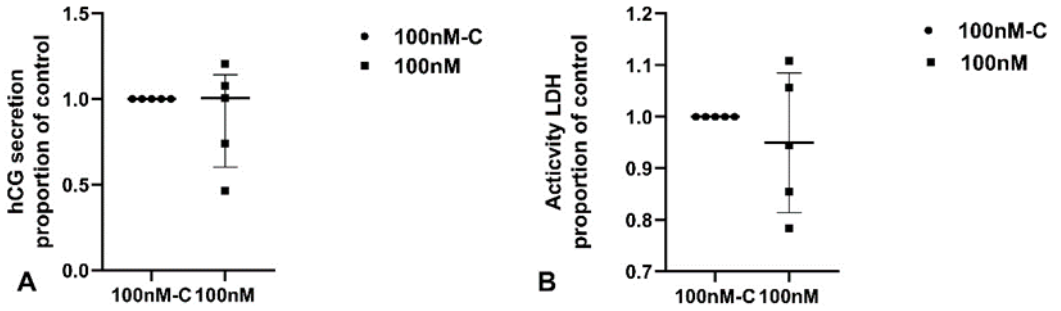


Figure 5.3 hCG and LDH levels in the conditioned culture medium from the explants incubated with 100nM miR-377 mimics or non-targeting control.

[A] hCG secretion in the culture medium from at day 4. [B] LDH levels in the culture medium from the explants transfected with miR-377 mimics or non-targeting control. Median+/-IQR; one sample Wilcoxon test.

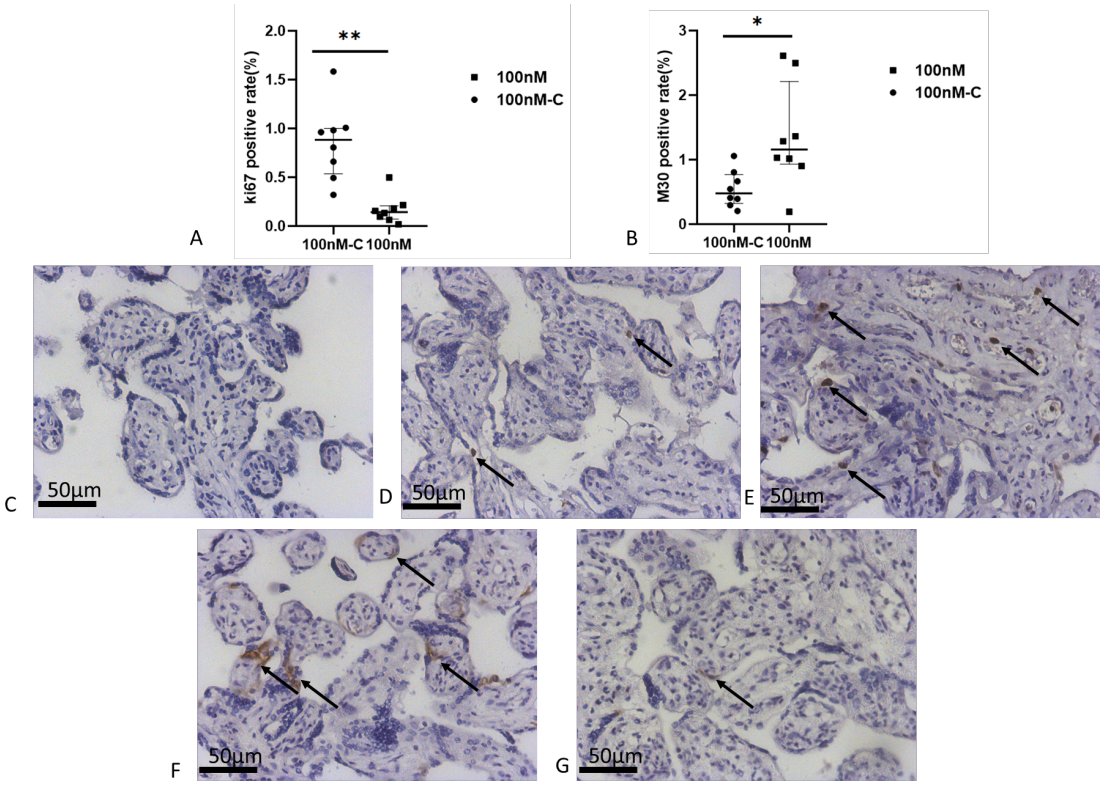


Figure 5.4 Immunostaining for Ki-67 or M30 in the cultured villous explants.

[A] and [B] Graphs of quantification of Ki-67 and M30 staining, respectively. [C] Negative control with the non-immune mouse IgG. [D-E], Ki-67; [F-G], M30. The explants transfected with miR-377 mimics [D] [F] or non-targeting control [E][G]. Median+/-IQR; Wilcoxon test. *: P<0.05; **: P<0.01.

5.4 Discussion

This study identified potential upstream miRNA regulators of *ESRRG* in the human term placenta, it then investigated whether miR-377, one potential upstream mediator, could signal through *ESRRG* to influence placental cell turnover, to determine whether it can be implicated in the pathogenesis of placental dysfunction.

5.4.1 Regulation of *ESRRG* by miR-377 in the human placentas

Some studies have already identified miRNAs upstream of *ESRRG* in trophoblast-like cells and cancer cell lines (Eichner et al., 2010, Lu et al., 2015, Gao et al., 2018, Liu et al., 2018). Two studies found that the overexpression of miR-320a repressed invasion, proliferation and migration, by modulating *ESRRG*, in the trophoblastic cell line, HTR-8SV/neo (Gao et al., 2018, Liu et al., 2018). Our previous study also used HTR-8/Svneo cells and identified miR-424 as a potential regulator of *ESRRG* (Zou et al., 2019). As these studies all used the HTR-8SV/neo cell line, which is derived from a first trimester placenta, it is not truly representative of intact term placental tissue and cannot be used to assess the normal physiological functions of the placenta *ex vivo.* Thus, it is important to use a human term placental explant model to further our understanding of the regulatory pathways of *ESRRG* in the placenta.

In our previous project, we demonstrated that miR-377 targeted the mitogenic signalling pathway in the first trimester villous explants and overexpression of miR-377 reduced cytotrophoblast proliferation (Farrokhnia et al., 2014). As we observed reduced ESRRG protein expression in these first trimester explants, it is most likely that miR-377 is involved in the regulation of *ESRRG*. In the current study, we demonstrated that overexpression of miR-377 reduced the mRNA and protein level of ESRRG in the cultured explants from third trimester as well. As the regulatory pathways of miRNA are complex, and a single miRNA can inhibit hundreds of downstream genes (Selbach et al., 2008), this suggests that miR-377 could target other downstream genes within the placentas. Therefore, the existing evidence suggests that *ESRRG* may be a target gene of miR-377 in the placenta.

However, miR-377-mediated knockdown of *ESRRG* did not affect expression of four of the downstream effectors of *ESRRG*. This may be due to the short-term culture of the

explants with the miRNA mimics: as the villous explants were only cultured for 48 hours, this might not allow enough time for the reduction in expression of *ESRRG* to affect expression of its downstream effectors. Further research is needed using a long-term explant culture to determine the precise downstream effects of miR-377 overexpression on *ESRRG* signalling. Moreover, as there are more downstream genes than the four genes which were modulated by *ESRRG* (Zou et al., 2021), it is also possible that the miR-377-*ESRRG* signalling pathway targets other downstream effectors and affects other aspects of placental cell function. Further research is needed to explore the expression of other potential downstream effectors.

5.4.2 The effects of miR-377-*ESRRG* signalling on trophoblast proliferation and apoptosis

Disruption of placental cell turnover i.e., increased apoptosis and decreased proliferation is observed in FGR (Heazell et al., 2008, Heazell et al., 2011). As observed in our previous study in first trimester placenta (Farrokhnia et al., 2014), we found that overexpression of miR-377 reduced the numbers of cells in the cell cycle in term villous explants. Similarly, overexpression of miR-377 also increased the number of apoptotic cells, a phenomenon that has not previously been reported. Given that several studies have explored the possibilities of using miRNAs as therapeutics to correct aberrant signalling in mice (Beards et al., 2017), this may provide a therapeutic avenue for preventing abnormal placental cell turnover by manipulating the miR-377-*ESRRG* axis. However, because the downstream genes of *ESRRG* did not change in this study, this cannot exclude that the effect of miR-377 on number of cells in cycle is mediated independently of *ESRRG*. Further research is also needed to clarify whether miR-377-*ESRRG* signalling can influence other mitogenic signalling pathways.

No alteration of hCG secretion or LDH release was observed in the explants transfected with miR-377 mimics or non-targeting control, suggesting that the miR-377-*ESRRG* axis does not affect syncytiotrophoblast differentiation or cell necrosis.

5.4.3 Upstream miRNAs expression in FGR placentas

No previous studies have explored the expression level of miR-377 in the FGR placentas; in this study, we did not observe any significant change in expression of

miR-377 in our samples. However, we have demonstrated that miR-377 contains a *ESRRG* binding site and we have previously shown that expression of *ESRRG* is reduced in FGR placentas. Thus, we cannot rule out whether dysregulation of *ESRRG* signalling in FGR placentas is mediated, at least in part, by miR-377. Moreover, the definition of FGR used in this study is an IBR less than 5th centile; miR-377 expression may be related to the severity of FGR, so increased miR-377 expression may only be observed in pregnancies with the lowest IBRs. In this study, there are only 9 FGR placentas and all pregnant women were of White British ethnicity. Whether the expression of miR-377 is associated with the ethnicity of pregnant women is still unclear and further research with a larger sample size is needed to determine the relationship between miR-377 expression and FGR placentas in the future. Alternatively, miR-377 may regulate *ESRRG* independently of changes in its own expression level.

Other miRNAs, including miR-320a and miR-424, have been identified as upstream miRNAs (or potential upstream miRNAs) of *ESRRG* in HTR-8/Svneo cells, and have been shown to alter their proliferation, migration, and invasion (Liu et al., 2018, Gao et al., 2018, Zou et al., 2019). Further investigation of the potential regulatory relationships between miR-320a, miR-424 and *ESRRG* in a more physiological model, cultured villous explants, will be helpful to better understand *ESRRG* signalling in the human placenta.

5.4.4 Strengths and Limitations

This study used a cultured term villous explant model which is a more physiologically relevant, 3D culture model, compared to trophoblastic cell lines and primary cells. Also, this study used a more convenient transfection method in the explant model, and this further confirms this method is effective to transfect the miRNA mimics. There are some limitations of this study; firstly, we only used short-term explant cultures, which might not be long enough to determining the downstream effects of miR-377 overexpression. Long-term cultures are required to explore whether miR-377 could alter *ESRRG*'s downstream gene expression. The limitations of this study are the extremely decreased ESRRG protein in the cultured explants. In addition, ESRRG protein is mainly localised in the trophoblast in the fresh placental tissue but mainly localised in the stromal cells in the cultured villous explants, and different protein

localisation may limit the exploration of *ESRRG* signaling pathways in this model. Other *in vivo* or *in vitro* models need to be used in future work to study these pathways, like organoids. Moreover, this study only recruited limited number of placentas, and further research with a larger samples size is also needed.

5.5 Conclusions`

In conclusion, overexpression of miR-377 in cultured term villous explants reduced the mRNA and protein levels of ESRRG, but did not alter the mRNA levels of *ESRRG*'s downstream genes. However, miR-377 overexpression reduced cell proliferation and apoptosis in these explants. The signalling pathways of miRNAs and *ESRRG* may be involved in the mediation of cell turnover in the placenta which strengthens the need to understand the role of miRNAs in the pathogenesis of FGR.

Acknowledgments

The authors thank the midwives for recruiting the placentas for us and thank all the pregnant women who donated their placentas to this study.

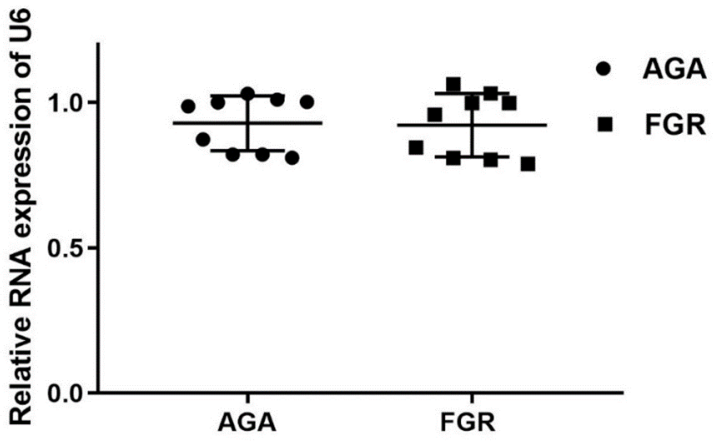
Supplementary Table 5.1 Participant demographics of AGA or FGR pregnancies.

Characteristic	AGA controls (n=9)	FGR (n=9)	P value
Birthweight (g)	3441.0±322.6(3020-3930)	1948.9±687.3 (717-2710)	<0.0001****
Birthweight percentile	51.0±21.2 (21.2-79.6)	1.4±1.4 (0-3.5)	<0.0001****
Fetal sex (female/male)	5/4	7/2	NS
Gestational age at delivery (weeks)	39.0±0.8 (37.0-40.5)	36.2±3.0 (30.3-39.4)	0.006**
Ethnicity			
White British	9	9	
Maternal age (years)	34.8±2.7 (30.0-39.0)	31.0±5.0 (20.0-39.0)	NS
Gravidity	2±1 (1-4)	3±1 (2-6)	NS
Parity	1±1 (0-2)	1±1 (0-3)	NS
Nulliparous (%)	4 (44.4%)	1 (11.1%)	0.03*
BMI (kg/m2)	29.0±5.2 (22-42)	25.7±5.7 (18-36)	NS

The result was shown as mean ± SD or median ± IQR with range, NS, no significant, BMI, body mass index. * P<0.05; **P<0.01, ***P<0.001, ****P<0.0001. AGA, appropriate for gestational age; FGR, fetal growth restriction.

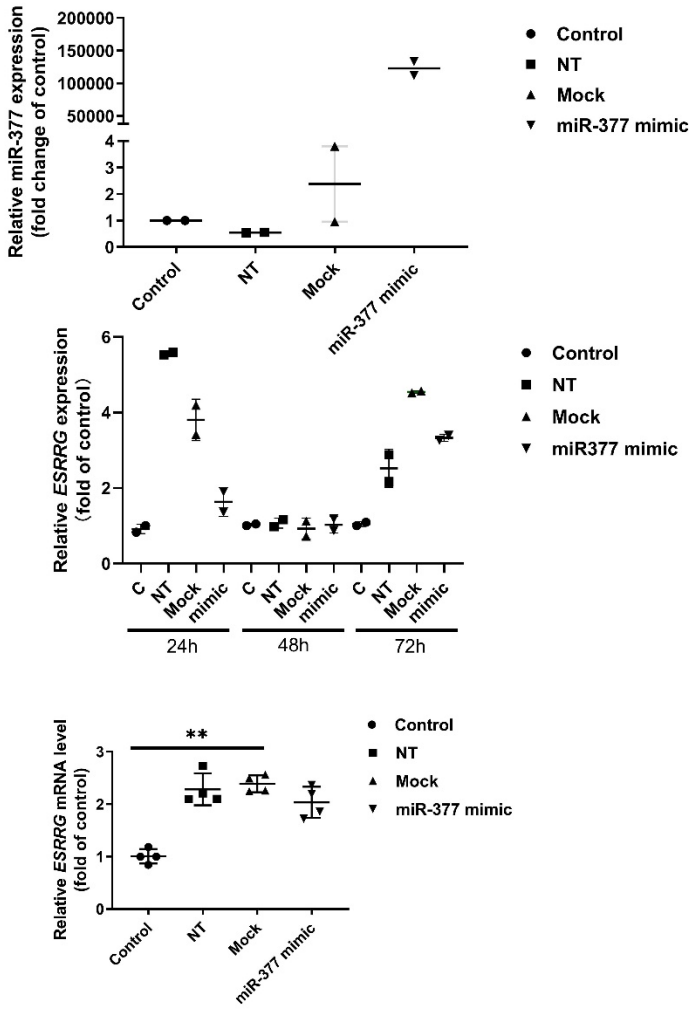
Primer set	Sequence
RPLP0	Forward: 5'-TGC ATC AGT ACC CCA TTC TAT CA-3'
	Reverse: 5'-AAG GTG TAA TCC GTC TCC ACA GA-3'
ESRRG	Forward: 5'-CTG ACG GAC AGC GTC AAC C-3'
	Reverse: 5'-GGC GAG TCA AGT CCG TTC TG-3'
HSD17B1	Forward: 5'-GCC TTC ATG GAG AAG GTG TT-3'
	Reverse: 5'-CGA AAG ACT TGC TTG CTG TG-3'
CYP191.1	Forward: 5'-ACG GAA GGT CCT GTG CTC G-3'
	Reverse: 5'-GTA TCG GGT TCA GCA TTT CCA-3'
PLAC1	Forward: 5'-ATT GGC TGC AGG GAT GAA AG-3'
	Reverse: 5'-TGC ACT GTG ACC ATG AAC CA-3'
HSD11B2	Forward: 5'-GAC CTG ACC AAA CCA GGA GA-3'
	Reverse: 5'-GCC AAA GAA ATT CAC CTC CA-3
U6	GGGCAGGAAGAGGGCCTAT
hsa-miR-377	AUCACACAAAGGCAACUUUUGU
hsa-miR-204-5p	UUCCCUUUGUCAUCCUAUGCCU
hsa-miR-30a-5p	UGUAAACAUCCUCGACUGGAAG
hsa-miR-26a-5p	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-23-3p	AUCACAUUGCCAGGGAUUUCC
hsa-miR-34b-5p	UAGGCAGUGUCAUUAGCUGAUUG
hsa-miR-30e	UGUAAACAUCCUCGACUGGAAG

Supplementary Table 5.2. Primer sequence of *ESRRG* and its downstream genes and miRNA's target sequence. *ESRRG*, oestrogen related receptor gamma; *HSD17B1*, hydroxysteroid 17-beta dehydrogenase 1; *CYP191.1*, cytochrome P-450; *PLAC1*, placenta-specific 1; *HSD11B2*, hydroxysteroid 11-beta dehydrogenase 2, miRNA, microRNA.



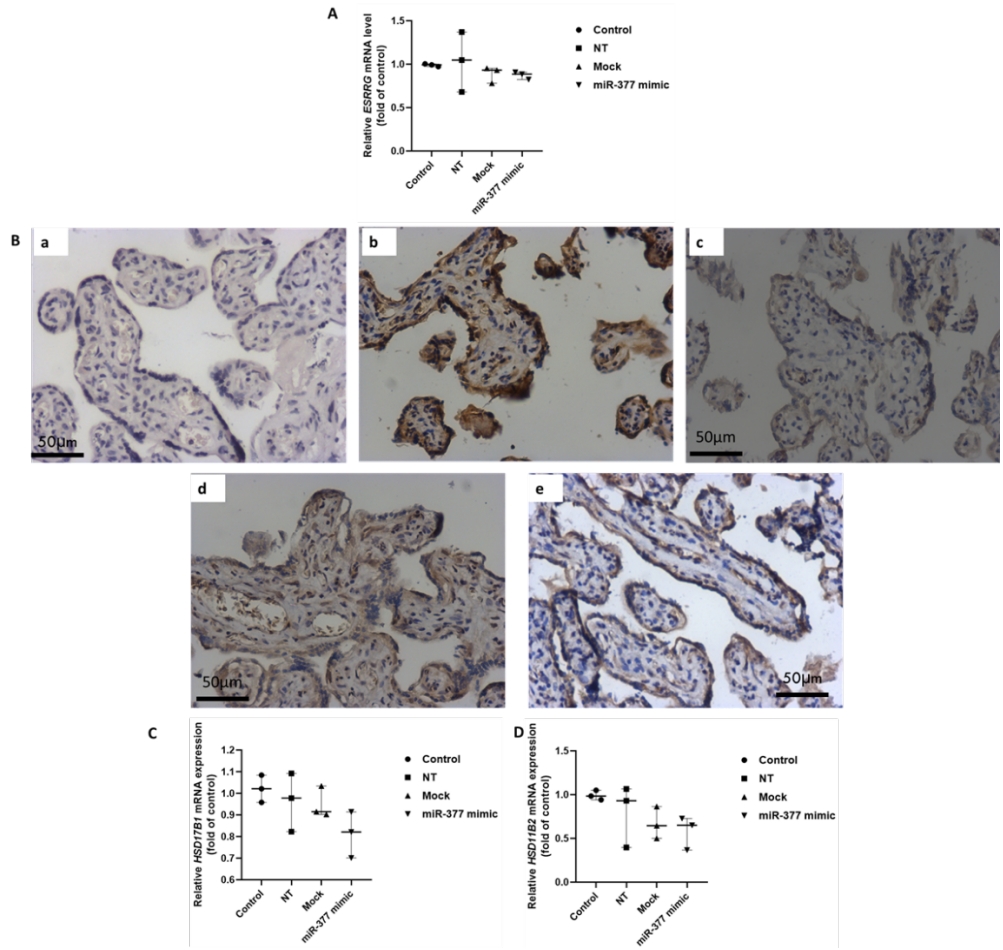
Supplementary Figure 5.1. The RNA expression of *U6* in the AGA and FGR placentas.

The RNA expression of *U6* was stable in the human placentas. Median +/-IQR; Mann-Whitney U test; n=9. FGR, fetal growth restriction, AGA, appropriate for gestational age.



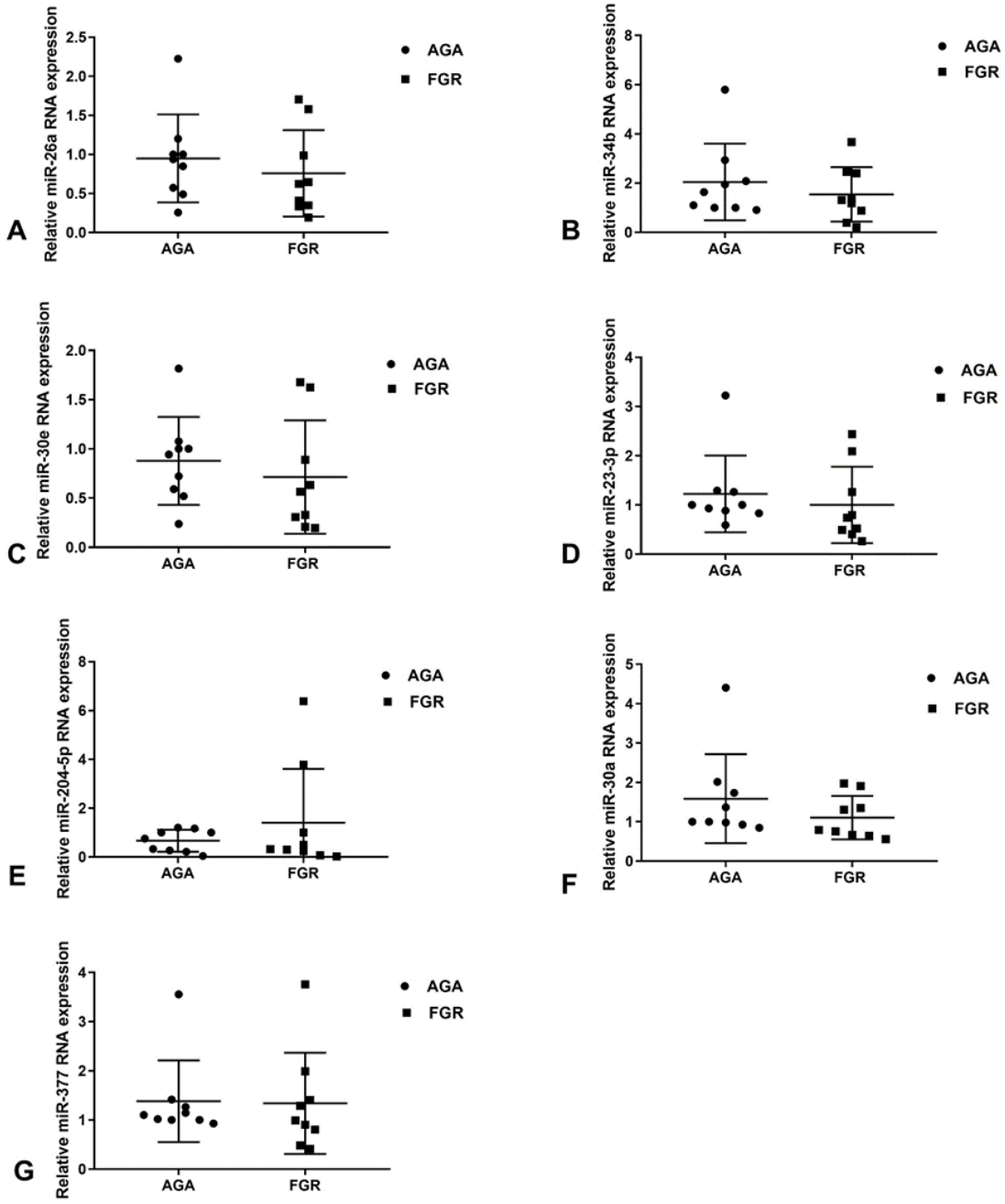
Supplementary Figure 5.2. The time course of miR-377 mimic in BeWo cells.

[A], The RNA levels of miR-377; BeWo cells were cultured for 48 hours and transfected with miR-377mimics (40nM), N=2. [B], BeWo cells were transfected with 40nM miR-377 mimics by using transfection reagents, which were harvested at 24, 48, or 72 hours of culture, N=2. [C], The mRNA levels of *ESRRG* in the BeWo cells transfected with 40nM miR-377 mimics or non-targeting mimics for 48 hours, N=2; the RT-PCR results were repeated twice. Control, untreated control; NT, non-targeting control (40nM); Mock, with transfection reagents; mimic, 40nM miR-377 mimics. Median+/-IQR. Friedman test.

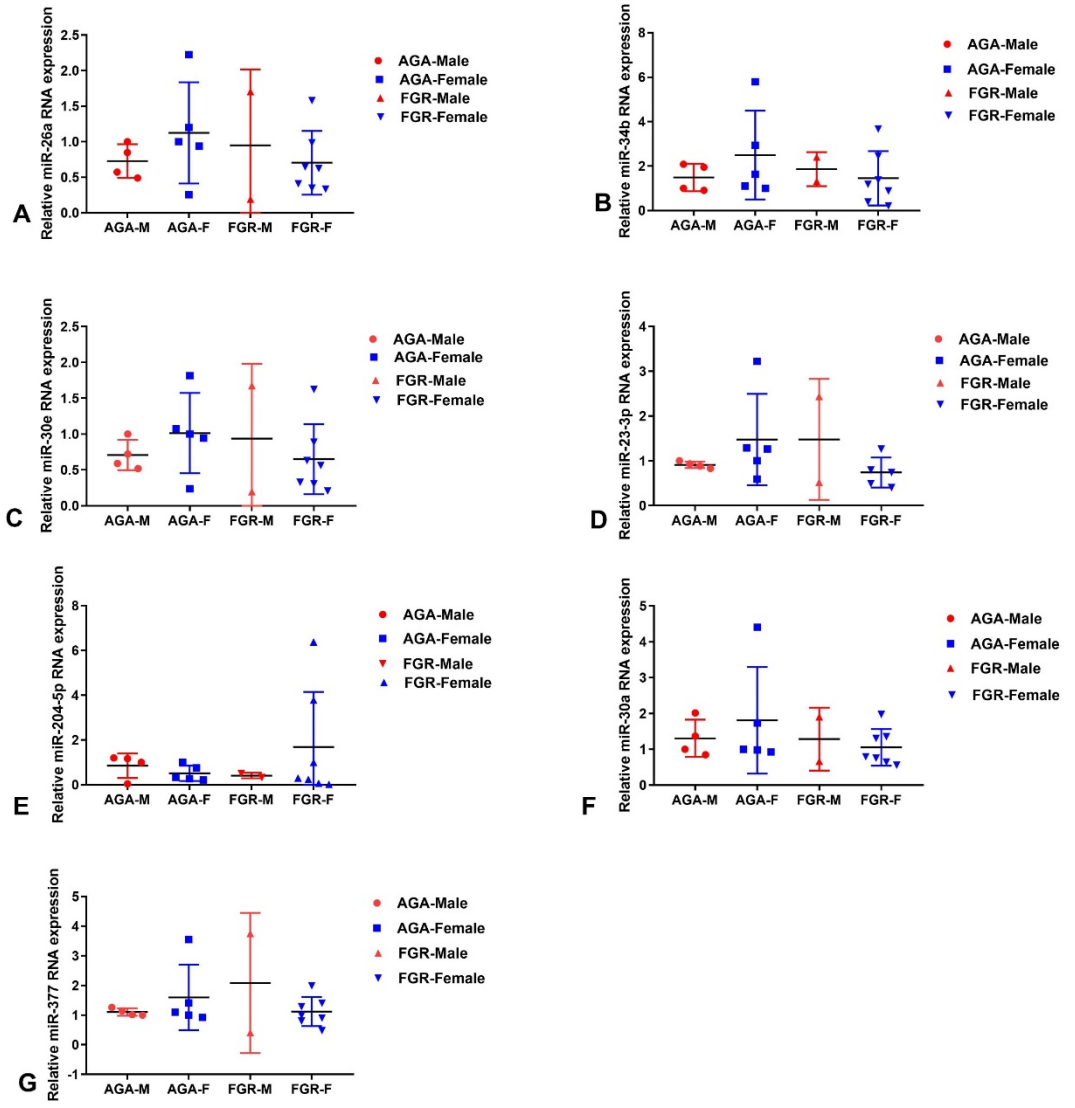


Supplementary Figure 5.3. Expression of *ESRRG*, *HSD17B1*, and *HSD11B2* in villous explants transfected with miR-377 mimic or non-targeting control with transfection reagents and cultured for 48 hours.

[A] mRNA levels of *ESRRG*; [B] The representative images of ESRRG protein staining in the explants transfected with miR-377 mimics or miRNAs nontargeting control; negative control is applied with non-immune rabbit IgG without primary antibody. Control, untreated control (b); Mock, explants transfected with transfection reagents alone (c). NT, non-targeting control (26.7nM) (d); mimic, miR-377 mimics (26.7nM) (e). [E] and [F], the mRNA levels of downstream genes, *HSD17B1* (hydroxysteroid 17-beta dehydrogenase 1), and *HSD11B2* (hydroxysteroid 11-beta dehydrogenase 2). Friedman test; median ± IQR, n=3.



Supplementary Figure 5.4. Expression of 7 potential upstream miRNAs of *ESRRG*. Figures 1 A-H show the RNA expression of miR-26a, miR-34b, miR-30e, miR-23-3p, miR-30a, miR-377, and miR-204-5p in FGR and AGA human term placentas. The black dots represent the AGA group, and the black squares represent the FGR group. Median +/-IQR; Mann-Whitney U test.



Supplementary Figure 5.5. Expression of miRNAs potentially upstream of *ESRRG* in term FGR and AGA placentas, divided by fetal sex.

A-G shows the RNA expression of miR-26a, miR-34b, miR-30e, miR-23-3p, miR-30a, miR-377, and miR-204-5p in male or female FGR and AGA placentas. Mean+/-SD; Kruskal-Wallis test. AGA-M, AGA male placentas; AGA-F, AGA female placentas; FGR-M, FGR-male placentas; FGR-F, FGR-female placentas. AGA, appropriate for gestational age; FGR, fetal growth restriction. *: P<0.05.

6 CHAPTER 6: General Discussion

Overview

FGR is an important complication of pregnancy, not only because it is associated with a high risk of stillbirth and neonatal death, but it also predisposes to long-term complications, including abnormal neurodevelopment, cardiovascular diseases, and metabolic disorders in the offspring (Gardosi et al., 1998, Bernstein et al., 2000, Baschat, 2011, Gardosi et al., 2013, Ramirez-Velez et al., 2017, Crispi et al., 2018, Pels et al., 2019). While impaired placental function is proposed to cause a significant proportion of cases of FGR there is no effective therapies to improve placental function thereby preventing or treating FGR, (Veerbeek et al., 2014, Spinillo et al., 2015, Spinillo et al., 2019). Although dysregulation of some placental signalling pathways is seen in FGR, such the insulin-like growth factor receptor, p53 and *ESRRG* signalling pathways (Abuzzahab et al., 2003, Forbes et al., 2008, Forbes and Westwood, 2008, Heazell et al., 2011, Zhu et al., 2018a, Harris et al., 2019), the mechanisms of placental dysfunction underlying FGR are not fully understood; this knowledge is critical to develop a therapeutic intervention for FGR pregnancies.

Impaired remodeling of the maternal spiral arteries has been hypothesized to cause the hypoxic conditions which are observed in FGR placentas (particularly in severe early-onset cases), and lead to deficient trophoblast function, including reduced proliferation, elevated trophoblast apoptosis, decreased cytotrophoblast differentiation, and increased necrosis (Heazell et al., 2008, Tal et al., 2010, Heazell et al., 2011, Robb et al., 2017). Meanwhile, low fetal weight, SGA, or FGR has been observed in the pregnancies with maternal BPA exposure, predominantly affecting the male infants (Chou et al., 2011, Snijder et al., 2013, Troisi et al., 2014), although the sex-specific effects of BPA in FGR pregnancies are still unclear. Altered miRNA signalling has also been attributed to the placental dysfunction observed in FGR (Huang et al., 2013).

Hypoxia, BPA, and miR-320a have all been identified as upstream regulators of *ESRRG* in trophoblastic cell lines, or cancer cell lines (Kumar and Mendelson, 2011, Zhang et al., 2016, Liu et al., 2018, Gao et al., 2018, Yaguchi, 2019), but the role of placental *ESRRG* signalling in the placental dysfunction underlying FGR is still unknown. Therefore, the hypothesis tested here was that placental *ESRRG* signalling contributed

to the pathophysiology of FGR, and deficient *ESRRG* expression, which was mediated by its upstream regulators, affects placental function by directly modulating its downstream effectors. In healthy placentas, ESRRG is highly expressed in syncytiotrophoblast; meanwhile, both the mRNA and protein expression of ESRRG is significantly decreased in the syncytiotrophoblast of placentas complicated by FGR (Kumar and Mendelson, 2011, Poidatz et al., 2012, Poidatz et al., 2015a, Zhu et al., 2018a). As syncytiotrophoblast is a barrier on the outside and cytotrophoblast is underneath, the link between syncytiotrophoblast and cytotrophoblast cannot be completely separate, and gene expression changes in syncytiotrophoblast could further influence cytotrophoblast function. For example, one previous study suggested that IGF signalling pathways in syncytiotrophoblast influence cytotrophoblast proliferation (Forbes et al., 2008). In addition, siRNA-mediated knockdown of *ESRRG* leads to impaired proliferation and invasion of the HTR-8/SVneo cell line, suggesting that *ESRRG* signalling may contribute to the development of pathology underlying FGR (Zhu et al., 2018a). Since placental hypoxia, BPA exposure, and altered miRNA expression serve as possible key regulators of placental dysfunction in FGR, and *ESRRG* expression can be regulated by each of these factors, we hypothesised that *ESRRG* may be one possible "missing link" between these risk factors and placental dysfunction. Exploring placental *ESRRG* signalling and the effects of altered *ESRRG* signalling on placental function may be helpful to understand the pathology of FGR, and identify new therapeutic targets.

6.1. Major findings of the study

6.1.1 Hypoxic regulation of *ESRRG*

This study measured the expression of *ESRRG* and its downstream genes in FGR and AGA placentas (Chapter 3). Both the mRNA and protein levels of ESRRG, and the mRNA levels of its downstream genes were significantly decreased in the stromal cells and villous trophoblast of placentas complicated by FGR. In this study, placental explants were cultured in 1% O_2 to mimic hypoxia and phenotypic changes common to those observed in FGR were noted, including decreased cytotrophoblast proliferation and syncytiotrophoblast differentiation and increased apoptosis and necrosis (Macara et al., 1996, Levy et al., 2002, Heazell et al., 2008, Heazell et al., 2011). Culture under hypoxic conditions significantly decreased the expression of *ESRRG* and its downstream genes, consistent with the observation of reduced *ESRRG* expression in FGR placentas. In the studies reported here reduced expression of *ESRRG* correlated with reduced expression of *HSD11B2* and *PLAC1* in the cultured villous explants. In addition, exposure of hypoxic explants to an agonist of *ESRRG*, DY131, rescued the reduced *ESRRG* expression and restored the impaired cell turnover observed. These findings suggest that DY131 may represent a novel therapeutic intervention to increase the level of *ESRRG* in FGR.

6.1.2 The sex-specific effects of BPA on *ESRRG* signalling pathways

Exposure to the environmental pollutant BPA is related to a sex specific reduction in birthweight, disproportionately affecting male infants (Chou et al., 2011, Snijder et al., 2013). *ESRRG* is a specific receptor for BPA in the human placenta and BPA binds to *ESRRG* with a 800 to 1000-fold higher affinity than to other receptors (Takayanagi et al., 2006, Liu et al., 2007, Takeda et al., 2009, Liu et al., 2012, Liu et al., 2019). However, only one study to date has investigated the effect of BPA-induced *ESRRG* signalling on trophoblast function and reported that it inhibited proliferation of the trophoblastic cell line, JEG-3 (Morice et al., 2011).

Placental BPA concentration has been reported to be 0.53 - 280 ng/g (equivalent to 2.3pM-1.2nM) (Schonfelder et al., 2002, Cao et al., 2012, Lee et al., 2018). Though evidence supports the relationship between low fetal weight and BPA exposure, BPA

levels were mainly measured in the maternal urine, maternal blood serum, and/or fetal cord serum samples instead of placental tissue (Chou et al., 2011, Snijder et al., 2013, Miao et al., 2011). Our experiments found levels of BPA in the placenta to be consistent with previous studies and my data may explain the sex-specific effects of BPA on the fetal weight underlying placental exposure, physiological BPA exposure (1nM) reduced *ESRRG* signalling in placental explants from male infants after 48 hours of culture, but did not affect placentas from female neonates.

As discussed in Chapter 3, impaired *ESRRG* signalling pathways led to placental dysfunction which is consistent with previous studies; BPA exposure damages trophoblast proliferation and invasion of a trophoblast cell line (Morice et al., 2011, Wang et al., 2015). In addition, the results in Chapter 4 have shown that *ESRRG* expression was increased following treatment with a higher BPA exposure than physiological levels (1µM BPA) for 24 hours, mainly in the female infants, which is consistent with previous studies using female cell lines. These data on the sex-specific effects of BPA may indicate placentas from male infants are more sensitive to BPA exposure, but we should note that because the related studies are still limited, future studies are needed to further confirm or refute the safety of BPA in both maternal and fetal side, and to explore the sex-specific effects of BPA.

Furthermore, there was no significant difference in the percentage of proliferating and apoptotic cells, hCG secretion, or LDH release in BPA treated explants, compared to controls. This suggested that acute BPA exposure at the concentrations tested did not alter placental cell turnover or differentiation. However, as described in Chapter 5, more proliferative and apoptotic markers will be measured in the further work to confirm the effects of BPA on the trophoblast turnover.

6.1.3 The regulatory relationship between miR-377 and *ESRRG*

miR-320a has been identified as an upstream regulator of *ESRRG* in the trophoblast-like cell line, HTR-8/SVneo (Gao et al., 2018, Liu et al., 2018). In the current study, we sought to identify other miRNAs which regulated *ESRRG* and may be responsible for the abnormal trophoblast turnover observed in FGR. The predictive software, TargetScan, identified miR-377 as a possible upstream regulator of *ESRRG*, and high

levels of miR-377 have been shown to reduce cytotrophoblast proliferation in first trimester placental villous explants, via its downstream actions on mitogenic pathways (Farrokhnia et al., 2014). Therefore, we used the term villous explant model to explore the potential regulatory relationship between miR-377 and *ESRRG*. Overexpression of miR-377 in the term villous explants reduced both mRNA and protein levels of ESRRG, but it did not affect the mRNA expression of *ESRRG*'s downstream genes, including *HSD17B1*, *CYP191.1*, *HSD11B2*, and *PLAC1*. miR-377 RNA expression was comparable between the FGR placentas and AGA placentas, and exposure to hypoxia did not alter the mRNA levels of miR-377 in cultured explants. However, increased expression of miR-377 led to a reduction in proliferation and increased apoptosis within the explants; it is still unclear if these changes are mediated by or are independent of *ESRRG* signalling. In first-trimester placental explants, miR-377 acts via *MAPK1* and *MYC* to alter cell proliferation (Farrokhnia et al., 2014). Therefore, whether miR-377 acts directly through *ESRRG* to influence cell turnover in villous explants, or via its actions on *MAPK1* and *MYC*, is still unclear.

6.2 Mutual regulation of *ESRRG* by hypoxia, BPA and miRNAs

The effects of hypoxia, BPA and miRNAs on *ESRRG* expression and trophoblast function may not act in isolation, so possible interactions between these pathways should be considered. The schematic images of *ESRRG* signalling pathways are shown in Figure 6.1.

6.2.1 Hypoxia and maternal BPA exposure

In the mouse, maternal BPA exposure (50 μg/kg or 4μM) in pregnancy can cause impaired remodeling of uterine spiral arteries, which leads to intermittent uteroplacental blood flow and placental hypoxia (Muller et al., 2018, Ye et al., 2019). Pregnancy complications, including FGR and preeclampsia are characterized by impaired uteroplacental perfusion and placental hypoxia (Muller et al., 2018, Ye et al., 2019), thus exposure to BPA in early pregnancy may lead to placental hypoxia later in gestation.

Although these studies in mice did not measure local oxygen levels or markers of hypoxia in the placentas, it has previously been reported that BPA can modulate HIF-

1alpha protein expression in BeWo cells and breast cancer cell lines, suggesting complex interrelationships between these signalling pathways (Ponniah et al., 2015, Xu et al., 2017). Conflicting information is available of the effects of BPA on HIF-1alpha. One study reported that BPA exposure (9µM) reduced HIF-1alpha protein expression in BeWo cells (Ponniah et al., 2015); however another reported that exposure of the breast cancer cell lines SkBr-3 and MDA-MB-231 to 1µM BPA under hypoxic conditions increased HIF-1alpha protein expression (Xu et al., 2017). Other publications have revealed that 200µM BPA can induce HIF-1alpha protein degradation in the human hepatocarcinoma cell line, Hep3B cultured in 1%O_2 (Kobayashi et al., 2018). These findings suggest that the effects of BPA on hypoxia signalling pathways are complex and lower dose BPA (1µM) exposure might be related to higher HIF-1alpha expression. Since BPA exposure can also modulate invasion, proliferation, and migration of trophoblast cell lines (Wang et al., 2015, Lan et al., 2017, Ye et al., 2019), the combined effects of BPA exposure, hypoxia and *ESRRG* signalling on different aspects of trophoblast functions are still unknown, and whether BPA and hypoxia synergistically modulate the expression of *ESRRG* in the human placenta still needs to be determined.

4-hydroxytamoxifen (4-OHT) is an inverse agonist which can suppress the intrinsic transcriptional activity of ESRRG (Chao et al., 2006). DY131 is phenolic acyl hydrazines, which is a potential ligand for ESRRG and it increases the basal activity of ESRRG (Yu and Forman, 2005). BPA can bind to human ESRRG with high affinity and acts as an antagonist of the inverse agonist activity of 4-OHT on ESRRG (Takayanagi et al., 2006). Therefore, the mechanism between DY131 and BPA to activate ESRRG expression is different. There are no previous studies to explore the relationship between BPA and DY131, and it is still unclear whether there is any correlation between BPA and DY131 in the human placenta.

6.2.2 Hypoxia and miRNAs

Although the regulatory relationship between miR-377 and *ESRRG* was not explored in this study, it is still critical to consider the effect of hypoxia on the expression of miRNAs previously reported to regulate *ESRRG* expression. Reduced expression of miR-377 has been reported in mesenchymal stem cells cultured in 1% O_2 (Wen et al., 2014); however, I did not observe reduced expression of miR-377 in villous explants

cultured in hypoxic conditions (Chapter 5). Therefore, I can exclude hypoxia as a regulator of miR-377 in term placental explants. However, other miRNAs upstream of *ESRRG* could be modulated by hypoxia. As one example, miR-320a can target the 3'UTR of *ESRRG,* and has been shown to influence the proliferation, invasion, and migration of HTR-8/SVneo cells (Gao et al., 2018, Liu et al., 2018). miR-320a also can target HIF-1alpha in cancer-associated fibroblasts, confirmed by a dual-luciferase reporter assay (Zhang et al., 2020). Therefore, it is possible that *ESRRG* signalling might be regulated by hypoxia and miR-320a in human placentas.

6.2.3 Maternal BPA exposure and miRNAs

Although one study found no relationship between BPA exposure and altered placental miRNA expression by examining 654 miRNAs in 63 term placentas with a median BPA concentration of 264.9pg/g (equivalent to 1.2pM) (Li et al., 2015b), other studies suggest miRNAs can be affected by BPA exposure in mice or in trophoblastic cell lines; this thesis summarised the miRNAs which may serve a role in the BPA-*ESRRG* signalling pathways, including miR-377, miR-499-5p, and miR-146a etc. (Veiga-Lopez et al., 2013, Kovanecz et al., 2014, De Felice et al., 2015, Farahani et al., 2021).

6.2.3.1 miR-377

In penile tissue of male rats, low dose BPA (0.1mg/kg/day) induced miR-451-5p and reduced the expression of miR-377, miR-82, miR-203a, miR-347, and miR-328a, while higher BPA exposure (1mg/kg/day) downregulates miR-200, miR-203, and miR-205 (Kovanecz et al., 2014). According to the hypothesis explored in Chapter 5, miR-377 may be an upstream miRNA of *ESRRG,* but the results did not suggest the sex-specific effects of miR-377 expression in either the FGR and AGA placentas or cultured placental explants. Therefore, the relationship between miR-377, BPA and *ESRRG* signalling pathways need to be explored in future work.

6.2.3.2 miR-499-5p

BPA exposure can alter miRNA levels in both maternal and fetal tissue during pregnancy (Ma et al., 2020, Rasdi et al., 2020). In pregnant mice, BPA reportedly increased the expression of miR-499-5p in murine maternal heart tissue (Rasdi et al., 2020), and in humans, miR-499-5p was increased in both the circulation and in the

heart tissue from patients with heart failure (Corsten et al., 2010, Mohseni et al., 2018). Interestingly, placental miR-499-5p expression was significantly increased in women with preeclampsia, and in late onset FGR placentas (Hromadnikova et al., 2015a), but the level of miR-499-5p in the maternal circulation was decreased in SGA pregnancy (Kim et al., 2020). This suggests inconsistence in miR-499-5p expression between placentas and maternal serum. The regulation between miR-499-5p and *ESRRG* in human placentas is unknown, but one study suggests that *ESRRG* can promote expression of miR-499-5p in primary murine muscle cells (Gan et al., 2013). Since maternal circulating miRNAs could be related to placental dysfunction (Baker et al., 2021), maternal BPA exposure could target the miR-499-5p in circulation or in placenta, which may regulate *ESRRG* signalling pathways, but whether *ESRRG* regulates miR-499-5p, or whether *ESRRG* is one of the targets of miR-499-5p is unknown. The role of miR-499-5p in the BPA-*ESRRG* signalling pathway underlying placental dysfunction and FGR needs to be explored in future work.

6.2.3.3 miR-146a

Several other studies have reported that BPA exposure increased placental expression of miR-146 (Avissar-Whiting et al., 2010, De Felice et al., 2015). miR-146a expression was also increased in second-trimester placentas following maternal BPA exposure (De Felice et al., 2015); exposure of three placental cell lines (TCL-1, 3A, and HTR-8/SVneo) to BPA (110μM) for 6 days resulted in increased expression of miR-146a in 3A and HTR-8/SVneo cells. These studies all suggest the BPA exposure can alter the expression of several miRNAs in the human placenta or trophoblast-like cell lines. Although our study did not explore the effect of BPA on miRNA expression in the cultured villous explants, further work is needed to see whether an interaction is present.

6.2.3.4 Other miRNAs

One recent study in our lab found that miR-551a was reduced in the maternal serum of FGR pregnancies and also reported sex-specific differences in circulating maternal miRNAs in FGR, including miR-28-5p, miR-29c-3p, miR-301a-3p, miR-378a-3p, miR-409-3p, miR-454-3p, and miR-526-5p (Baker et al., 2021). As mentioned above, abnormal miRNA levels in maternal serum may be linked with placental dysfunction

in a sex-specific manner (Baker et al., 2021), so it is plausible that these circulating miRNAs may contribute to the impaired placental function underlying the BPA-related changes in FGR placentas. However, there are no available publications to test whether these sex-specific miRNAs are regulated by BPA. Although, miR-551a was down-regulated in the maternal serum of FGR pregnancies, and was also reduced in fetal mice ovaries following BPA exposure (umbilical serum BPA:11.5nM) (Veiga-Lopez et al., 2013, Baker et al., 2021). Whether miR-550a is related to BPA-*ESRRG* signalling pathways is another area for further work. Additional sex-specific differences in the regulation of *ESRRG* in response to hypoxia, BPA and miRNAs cannot be ruled out.

6.3 Limitations of this study

In this study, I applied two models to explore the regulatory relationship between *ESRRG* and its downstream genes, but were unable to successfully reduce the expression of *ESRRG* in either BeWo cells or placental explants using *ESRRG*-specific siRNAs within the timeframe of our experiments. The BeWo cell line originates from a choriocarcinoma and is used as a model of villous cytotrophoblasts, but one criticism of the use of trophoblastic cell lines is that they differ physiologically from primary cells (Kallol et al., 2018). In contrast to a cell line, placental villous explants are composed of multiple cell types and retain their 3-D structure. Although they are a more physiologically relevant model, their heterogeneity makes it more difficult to study the effects of *ESRRG* signalling on specific cell types. In addition, although high ESRRG mRNA and protein expression have been found in the fresh tissue in this study, ESRRG protein expression was significantly decreased in the cultured villous explants which may limit the exploration of ESRRG signaling pathways, such as the failure transfection with ESRRG siRNAs in the cultured villous explants. This could be overcome by using other models, including term primary cytotrophoblast cells, organoids, or a placenta-on-a-chip in the future (Kliman et al., 1986, Turco et al., 2018, Richardson et al., 2020). Moreover, this study only explored the effects of *ESRRG* signalling pathways in villous explants cultured for up to 4 days, so longer term culture is also needed to further understand effects which may require changes to transcription or maintenance of pathways.

Furthermore, this study only examined some of the many different trophoblast functions, including proliferation (Ki-67 staining), apoptosis (M30 staining), cell differentiation (hCG secretion), and cell necrosis (LDH release) in the cultured explants. It is possible that *ESRRG* could alter other trophoblast functions, including placental hormone metabolism, nutrient transporter expression and/or activity, or placental vascular structure and function, both *in vivo* and *in vitro*, so additional studies are needed. The expression of only four of *ESRRG*'s downstream genes, *HSD17B1*, *HSD11B2*, *CYP191.1*, and *PLAC1*, was measured, while there are some other potential downstream genes of *ESRRG* relating to mitochondrial or vascular function that are dysregulated in FGR placentas, like *VEGF*, *PDK4*, and *MCAD*. Expression of these genes has been studied in human primary cytotrophoblast and in mice; they could also be studied in our model system (Poidatz et al., 2012, Luo et al., 2014).

6.4 Perspectives and significance

The role of *ESRRG* signalling is critical in cardiovascular and metabolic disorders (Alaynick et al., 2010, Kim et al., 2012, Kim et al., 2019a, Vernier et al., 2020). First of all, this thesis tried to identify novel miRNAs upstream of *ESRRG*, and Chapter 5 showed that elevated expression of miR-377 can reduce the expression of *ESRRG* in cultured villous explants. This may provide another possible regulatory mechanism between miR-377 and *ESRRG* and explain the mechanism underlying abnormal cytotrophoblast turnover in explants with high miR-377 levels.

We have addressed our second hypothesis, that BPA-*ESRRG* signalling pathways are sex-specific in the human placenta. Our results showed that expression of *ESRRG* and its downstream genes are decreased in placental explants from male infants and increased in the female infants, which could partly explain the potential link between maternal BPA exposure and reduced fetal weight. BPA impaired the normal functions of trophoblast-like cells, and has recently been banned in neonatal products, like milk bottles, in high-resource countries (Benachour and Aris, 2009, Morice et al., 2011, Spagnoletti et al., 2015, Muller et al., 2018). The concentrations of BPA we used in this study (1pM to 1μM) have been widely detected in physiological samples, including maternal serum and human placental tissues (Ikezuki et al., 2002, Schonfelder et al.,

2002, He et al., 2009, Lukasiewicz, 2020). Thus, our results further support the safety concern of BPA exposure during pregnancy. Moreover, our data suggest that we should pay more attention to the BPA concentrations in maternal or neonatal samples in pregnancies complicated by FGR, especially those with male infants. It might be also important to measure the BPA concentration in maternal serum in areas known to have high BPA levels and reduce the usage of BPA-containing products during pregnancy.

As ESRRG protein is highly expressed in normal placentas, but impaired in placentas from pregnancies complicated by FGR and siRNAs mediated knockdown of *ESRRG* results in reduced proliferation and invasion in trophoblast-like cell lines, there is a potentially important role for *ESRRG* in the pathology of FGR (Zhu et al., 2018a). Improved knowledge of *ESRRG* signalling pathways and their role in the syncytiotrophoblast is critical to understand the physiology and pathophysiology of placental dysfunction in pregnancy complications like FGR. In this study, we confirmed that placental ESRRG mRNA and protein expression is reduced in pregnancies complicated by FGR and examined the potential upstream regulators and downstream genes of *ESRRG*. The results in this thesis confirm our overall hypothesis that hypoxia can modulate *ESRRG* signalling and alters cytotrophoblast turnover, which could contribute to the pathophysiology of FGR. We also demonstrated that the agonist of *ESRRG*, DY131, can rescue the hypoxia-mediated decrease in *ESRRG* signalling and restore the resulting aberrant cell turnover observed in the placental explants. Therefore, DY131 could be explored as a therapeutic drug to treat FGR pregnancies.

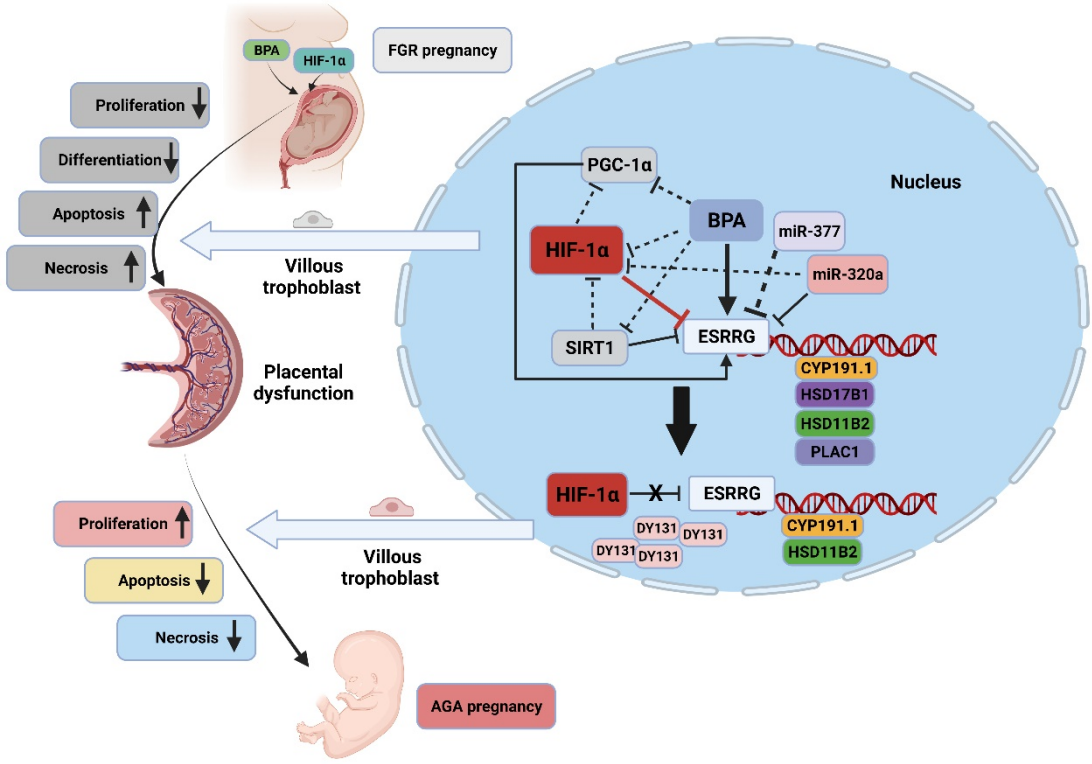


Figure 6.1 Schematic image of ESRRG signalling pathways. The dashed line is the regulation is not clear in the human placentas but validated in the animal models or cancer cell lines.

FGR, fetal growth restriction; AGA, appropriate weight for gestational age; *ESRRG*, oestrogen related receptor gamma; HIF-1alpha, hypoxia induced factor 1alpha; *HSD17B1*, hydroxysteroid 17-beta dehydrogenase 1; *CYP191.1*, cytochrome P450 family 19 subfamily a member 1; *HSD11B2*, hydroxysteroid 11-beta dehydrogenase 2; PLAC1, placenta-specific protein 1; BPA, bisphenol A; PGC-1alpha, peroxisome proliferator-activated receptor-gamma coactivator (PGC)- 1alpha; SIRT1, sirtuin 1.

6.5 Future work

6.5.1 Assessing other genes downstream of *ESRRG*

In this study, four genes downstream of *ESRRG* were identified; future work will investigate other possible regulatory relationships between *ESRRG* and *VEGFA*, and the potassium channels, *PDK4*, and *MCAD* as these genes have been identified as downstream targets of *ESRRG* in other systems, and are linked to aspects of placental dysfunction, such as abnormal angiogenesis or impaired trophoblast functions observed in FGR placentas (Zhang et al., 2006a, Bombail et al., 2010, Lee et al., 2012, Matsakas et al., 2012, Poidatz et al., 2012, Do et al., 2015).

6.5.1.1 *VEGFA*

Angiogenesis, which is mediated by growth factors, including VEGFA, is important to placental development and also serves a critical role in the development of FGR (Ahmed and Perkins, 2000, Arroyo and Winn, 2008, Gourvas et al., 2012). VEGFA protein is mainly localised in the stromal cells, syncytiotrophoblast, and decidual area (extravillous trophoblast) in the first trimester placental bed and is mainly localised in the stroma and syncytiotrophoblast in the second and third trimester placentas (Shiraishi et al., 1996, Zhou et al., 2002). Gene expression of *VEGFA* was upregulated in the placentas from FGR pregnancies (Szentpeteri et al., 2013). The potential regulatory relationship between *ESRRG* and *VEGFA* in the human placenta was considered because increased *Vegfa* mRNAs levels have been reported in the placentas of *Esrrg* (*Esrrg-/-*) knockout mice (Luo et al., 2014). *ESRRG* also targeted the promoter of *VEGFA* in rat primary mandibular condylar chondrocytes, assessed by ChIP assay, and in the rat retinal ganglion cell line (RGC-5), assessed via luciferase reporter assay (Do et al., 2015, Zhao et al., 2019). *VEGFA* is also involved in HIF-1 alpha mediated hypoxic signalling pathways, which is linked to PGC-1 alpha, a coactivator of *ESRRG* (Arany et al., 2008, Do et al., 2015). In this thesis, high HIF-1 alpha protein levels reduced ESRRG mRNA and protein expression in the explants cultured in 1% O_2 or with $CoCl_2$ treatment. Unlike the results in the Chapter 3, high expression of HIF-1 alpha increased ESRRG mRNA and protein expression in RGC-5 cells, with the induction of *VEGFA* mRNA expression, which was inhibited by an inverse agonist of *ESRRG*, GSK5182 (Do et al., 2015). This may be related to numerous differences between our

placental explant model and the rat retinal ganglion cell line. Although I did not observe evidence of regulation between *ESRRG* and *VEGFA* in HTR-8/SVneo cells (unpublished data), whether placental *VEGFA* is involved in HIF-1 alpha-*ESRRG* signalling pathways, or whether PCG-1alpha co-activates the expression of *ESRRG,* will be explored in other models, such as placental explants, primary cytotrophoblast cultures, organoids, or mouse models.

6.5.1.2 Potassium channels

In Chapter 1, I described how *ESRRG* induces mRNA and protein expression of the potassium channels KLK1, KCNQ1, KCNE1, KCNE3, and KCNE5 during primary cytotrophoblast differentiation, the effect of which was blocked by hypoxia (Luo et al., 2013). Although the sex-specific expression of potassium channels in the placenta is unknown, a few studies indicate that potassium channel expression is regulated by sex hormones (Song et al., 2001, Sakamoto and Kurokawa, 2019). For example, Kv4.3 channel expression was reduced in the rat myometrium following oestrogen exposure, and sex steroid hormones such as testosterone and 17β-oestradiol, have been shown to protect against ischaemia/reperfusion injury by directly activating mitochondrial potassium channels in cardiac myocytes (Song et al., 2001, Sakamoto and Kurokawa, 2019). These observations suggest that sex specific expression or activity of potassium channels may be observed in the placenta. To date, no study has explored the relationship between BPA and potassium channels expression or function in the placenta, but it has been shown in AD293 cells (derived from human embryonic kidney cells), primary human and canine coronary smooth muscle, and mice islets (Asano et al., 2010, Rottgen et al., 2014, Martinez-Pinna et al., 2019). Therefore, further work will assess whether BPA and/or miRNAs act through *ESRRG* to regulate potassium channel expression in the human placental models, such as villous explants.

6.5.1.3 PDK4 and MCAD

As mentioned in Chapter 1, *PDK4* and *MCAD* are potential downstream genes of *ESRRG* (Poidatz et al., 2012). Bisphenol analogues, but not BPA, have been shown to promote *PDK4* mRNA levels in human liver HL-7702 cells (Li et al., 2021). As the knowledge about the sex-specific expression of these two genes is limited, whether

these two downstream genes are involved in HIF-1 alpha, and/or BPA mediated *ESRRG* signalling pathways will be explored in placental explants in future work.

6.5.2 Assessing the mechanism of the sex-specific effects of BPA exposures

In BPA exposed placental explants, the mechanisms underlying the sex-specific regulation of *ESRRG* signalling pathways needs to be determined. UDP-glucuronosyl-transferases (UGT) can metabolise BPA in the human placenta (Shibata et al., 2002, Collier et al., 2002); as sex specific differences in UGT expression have been observed in the liver and kidney from humans and mice, the presence of sex-specific differences in placental UGT levels are still unclear (Shibata et al., 2002, Takeuchi et al., 2004, Shiratani et al., 2008, Buckley and Klaassen, 2009). Therefore, whether there is any difference in UGT expression and BPA metabolism in healthy placentas from male and female infants should be explored.

Another possible mechanism of placental sex-specific effects of BPA exposure may be related to epigenetic modulation, including methylation changes, and their effects on the levels of UGT enzymes. DNA methylation of the *ESRRG* gene and the expression of epigenetic regulators such as DNA methyltransferase-1 (*DNMT-1*) and *DNMT5*, could be measured in cultured villous explants.

Although BPA exposure has been linked to FGR pregnancies and reduced placental expression of *ESRRG* is associated with FGR in both our study and previous studies (Chou et al., 2011, Zhu et al., 2018a), it is still unclear whether there is a direct causal relationship between BPA exposure and the expression of *ESRRG* in FGR placentas. In the future, the mRNA and protein levels of ESRRG should be assessed in BPA-exposed FGR placentas and the presence of correlations between BPA concentration, fetal weight, and *ESRRG* expression should be analysed. In addition, a mouse or rat model of pregnancy could be used to explore the sex-specific effects of BPA on the *ESRRG* signalling pathways in the placenta. As only short-term explant culture (up to 48h) was used in this study, and no alterations in hCG secretion, Ki-67 and M30 staining were observed, longer-term explant cultures (i.e. 4–7 days) with BPA exposure could be assessed in the future work. Changes in the expression of more proliferative and

apoptotic markers, such as [^{3}H]-thymidine incorporation (proliferation), terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining (quantify apoptosis), and caspase activity assay (apoptosis), could be measured to assess the longer-term effects of BPA on trophoblast turnover.

6.5.3 Assessing the role of *ESRRG*'s co-activators or repressors in the regulation of *ESRRG*

Future studies should explore whether the co-activators or repressors of *ESRRG* are involved in the regulation of *ESRRG* in hypoxia or with BPA exposure. The co-activators, including PGC-1 alpha and PGC-1 beta, and the repressor SIRT1, should be measured in villous explants cultured under hypoxia or treated with BPA. In addition, the expression of *ESRRG* and its downstream genes could be detected after overexpression or silencing PGC-1 alpha, PGC-1 beta, or SIRT1 in term AGA placental explants, or primary cytotrophoblast cultured in hypoxia or treated with BPA. In addition, cytotrophoblast function in the cultured villous explants or primary cytotrophoblast will be measured, which will help understand if these co-regulators are involved in the regulation of *ESRRG* and identify whether these effectors alter cytotrophoblast function.

6.5.4 To assess the possible interaction between miR-377 and *ESRRG*

As I did not explore the exact nature of the regulatory relationship between miR-377 and *ESRRG* in the placenta, further experiments using a luciferase reporter assay are essential to understand whether miR-377 binds to the 3'UTR of *ESRRG*. In addition, it is important to assess whether alteration of cell turnover (changes in the basal rate of apoptosis and proliferation) is mediated directly through *ESRRG*, and not via other mitogenic signalling pathways as reported in first trimester explants (Farrokhnia et al., 2014). Further work could also test whether miR-377 could directly target *ESRRG* and regulate trophoblast turnover in first trimester explants. A miRNA-binding site target protector assay will be used to assess whether miR-377 could target *ESRRG,* to alter cytotrophoblast proliferation and apoptosis of cytotrophoblasts, independently from other than mitogenic pathways.

6.5.5 Assessment of the therapeutic potential of DY131

In this study, I observed that DY131, an agonist of *ESRRG*, can rescue altered cell turnover observed in explants cultured in hypoxia, via restoring the reduced *ESRRG* signalling. It would be helpful to confirm whether DY131 can reverse the placental pathological phenotypes in human placental explants from FGR pregnancies, including defective trophoblast function and reduced nutrient transport capacity, or other important aspects of placental function; this could be tested by applying DY131 to cultured explants from FGR placentas. Testing DY131 in a murine or non-human primate model of FGR, would help assess whether administration in early pregnancy could prevent FGR or whether administration upon diagnosis of FGR could be used to treat the pathology. In addition, as this thesis used placental explants from the third trimester, it is also critical to explore whether DY131 can alter cell turnover in cultured explants from first or second trimester placentas. These studies would allow us to collect important safety and efficacy data to inform clinical trials of DY131 in pregnancies with a high risk of FGR.

6.5.6 Assessing oestrogen metabolism (*CYP191.1*, *HSD17B1*), and cortisol metabolism (*HSD11B2*))

The level of hCG secretion from the syncytium of villous explants was measured in this study. There are other placental protein hormones, including human placental lactogen (hPL), progesterone, and pregnancy-associated plasma protein A (PAPP-A), which are secreted by the syncytiotrophoblast and whose levels change with placental dysfunction (Gardner et al., 1997, Fox and Chasen, 2009, Baker et al., 2017). Because the production of these protein hormones can be dysregulated in placental dysfunction (Gardner et al., 1997, Fox and Chasen, 2009, Baker et al., 2017), future work should include measuring the levels of these placental hormones in the conditioned culture medium of explants exposed to hypoxia or BPA to broaden understanding of how BPA affects placental dysfunction. This will enable assessment of whether these hormone levels are correlated to changes in *ESRRG* expression, and to understand if altered hormones expression is linked to *ESRRG*-related placental dysfunction.

The downstream genes of *ESRRG*, including *CYP191.1*, *HSD17B1*, or *HSD11B2*, are involved in the metabolism of placental oestrogen and cortisol (Benediktsson et al., 1993, Kamat et al., 2002), and low oestrogen levels and cortisol levels in the maternal serum has been found in the term pregnancies complicated by preeclampsia or FGR (Gardner et al., 1997, Wan et al., 2018, Jayasuriya et al., 2019), which suggest low placental oestrogen and cortisol level might be associated with FGR. In this thesis, I observed reduced gene expression of *CYP191.1* and *HSD11B2* in FGR placentas, and hypothesise that it might lead to reduced placental oestrogen and cortisol levels, as these two genes are responsible for oestrogen and cortisol metabolism (Benediktsson et al., 1993, Kamat et al., 2002). I observed reduced mRNA expression of *HSD11B2* and *CYP191.1* in explants exposed to both hypoxia and BPA. Therefore, it is essential to measure the oestrogen and cortisol concentrations in the conditioned cultured medium from these explants and assess whether hypoxia- or BPA-mediated *ESRRG* signalling influence oestrogen or cortisol metabolism in the placenta. Although I did not notice any alterations in proliferation or apoptosis in BPA-exposed placental explants, the possibility that the sex-specific effects of BPA-*ESRRG* signalling occur by altering placental hormone levels to further impair placental function needs further exploration.

6.6 Summary

This thesis provides further evidence for the impaired placental signalling pathways of *ESRRG* in FGR pregnancies. I applied an *in vitro* villous explant model to identify hypoxia-*ESRRG* signals, which can lead to reduced proliferation and elevated apoptosis in the human placenta. The *ESRRG* agonist, DY131, restored the impaired *ESRRG* signalling and reversed the hypoxic damage in the cultured villous explants, and could be developed as a potential therapeutic drug to treat placental dysfunction in FGR pregnancies in the future. In addition, *ESRRG* serves an important role in mediating both the placental effects of BPA exposure during pregnancy and explaining the sex-specific effects on the *ESRRG* signalling pathway. Thus, the observations from this thesis provide more understanding of the sex differences in neonatal pathology in the BPA-exposed FGR pregnancies.

7 Reference

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